

**Soybean blotchy mosaic virus:  
Molecular characterization and seasonal  
persistence**

by

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## **Declaration**

I, Elrea Strydom, hereby declare that this thesis submitted to the University of Pretoria for the degree PhD Microbiology contains my own work, and that the content contained within this thesis has not been submitted to any other university or institution.

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## List of Abbreviations

|            |                                                                                     |
|------------|-------------------------------------------------------------------------------------|
| A          | Adenosine                                                                           |
| AAU        | Association of African Universities                                                 |
| Acc number | Accession number                                                                    |
| ADV        | Alfalfa Dwarf Virus                                                                 |
| ag         | Attogram                                                                            |
| ANOVA      | Analysis Of Variance                                                                |
| ARC-PPRI   | Agricultural Research Council-Plant Protection Research Institute                   |
| B.C.       | Before Christ                                                                       |
| BLAST      | Basic Local Alignment Search Tool                                                   |
| BLASTn     | BLAST algorithm comparing a nucleotide query sequence against a nucleotide database |
| BNYV       | Broccoli Necrotic Yellows Virus                                                     |
| bp         | Base pairs                                                                          |
| BYSV       | Barley Yellow Striate Virus                                                         |
| C          | Cytosine                                                                            |
| °C         | Degrees Celsius                                                                     |
| cDNA       | Complementary Deoxyribonucleic Acid                                                 |
| CCoMV      | Cereal Chlorotic Mottle Virus                                                       |
| CCSV       | Cynodon Chlorotic Streak Virus                                                      |
| C:I        | Chloroform: Isoamyl alcohol                                                         |
| CMV        | Cucumber Mosaic Virus                                                               |
| CTAB       | Cetyltrimethylammonium Bromide                                                      |
| CVYV       | Croton Vein Yellowing Virus                                                         |
| DNA        | Deoxyribonucleic Acid                                                               |
| dATP       | Deoxyadenosine Triphosphate                                                         |
| dCTP       | Deoxycytosine Triphosphate                                                          |
| dGTP       | Deoxyguanine Triphosphate                                                           |
| dTTP       | Deoxythymine Triphosphate                                                           |
| dNTP       | Deoxynucleotide Triphosphate                                                        |
| dsRNA      | Double stranded RNA                                                                 |
| DTT        | Dithiothreitol                                                                      |
| DYVV       | Datura Yellow Vein Virus                                                            |
| EDTA       | Ethylenediaminetetraacetic Acid                                                     |
| Eds.       | Editors                                                                             |

|                   |                                                     |
|-------------------|-----------------------------------------------------|
| EMDV              | Eggplant Mottled Dwarf Virus                        |
| EST               | Expressed Sequence Tag                              |
| ER                | Endoplasmic Reticulum                               |
| et al.            | And others                                          |
| Exo I             | Exonuclease I                                       |
| F                 | Forward                                             |
| fg                | Femtogram                                           |
| FLSV              | Festuca Leaf Streak Virus                           |
| G                 | Guanine                                             |
| G                 | Glycoprotein                                        |
| g                 | Gravitational/centrifugal force                     |
| GPS               | Global Positioning System                           |
| GRI               | Genomics Research Institute                         |
| h                 | Hour                                                |
| H3                | Histone 3                                           |
| H <sub>2</sub> O  | Water                                               |
| HSD-test          | Honest Significant Differences-test                 |
| ICTV              | International Committee for the Taxonomy of Viruses |
| IEM               | Immunoelectron Microscopy                           |
| IMMV              | Iranian Maize Mosaic Virus                          |
| INE               | Inner Nuclear Envelope                              |
| IVCV              | Ivy Vein Clearing Virus                             |
| kb                | Kilo Bases                                          |
| KCl               | Potassium Chloride                                  |
| KOH               | Potassium Hydroxide                                 |
| L                 | RNA-dependent RNA polymerase protein                |
| LiCl              | Lithium Chloride                                    |
| LNyV              | Lettuce Necrotic Yellow Virus                       |
| LYMoV             | Lettuce yellow mottle virus                         |
| M                 | Matrix protein                                      |
| MFSV              | Maize Fine Streak Virus                             |
| MgCl <sub>2</sub> | Magnesium Chloride                                  |
| min               | Minutes                                             |
| ml                | Millilitre                                          |
| ML                | Maximum Likelihood                                  |
| mM                | Milimolar                                           |

|                    |                                                                          |
|--------------------|--------------------------------------------------------------------------|
| M-MLV              | Moloney Murine Leukemia Virus                                            |
| MMV                | Maize Mosaic Virus                                                       |
| mRNA               | Messenger Ribonucleic Acid                                               |
| mtCOI              | Mitochondrial Cytochrome c Oxidase subunit I                             |
| MYSV               | Maize Yellow Striate Virus                                               |
| N                  | Nucleoprotein                                                            |
| NaCl               | Sodium Chloride                                                          |
| NCMV               | Northern Cereal Mosaic Virus                                             |
| NCBI               | National Centre for Biotechnology Information                            |
| NJ                 | Neighbour Joining                                                        |
| NPC                | Nuclear Pore Complexes                                                   |
| NRF                | National Research Foundation                                             |
| nt                 | Nucleotide                                                               |
| ONE                | Outer Nuclear Envelope                                                   |
| ONNV               | O' Nyong-Nyong Virus                                                     |
| ORF                | Open Reading Frame                                                       |
| P                  | Phosphoprotein                                                           |
| <i>p</i> -distance | Pairwise distance                                                        |
| pg                 | Picogram                                                                 |
| pH                 | Log hydrogen concentration                                               |
| pss-RT-PCR         | Positive Strand Specific-Reverse Transcriptase-Polymerase Chain Reaction |
| Prof               | Professor                                                                |
| PVAS               | Plant Virus, Antiserum and Seroreagent collection                        |
| PVP                | Polyvinylpyrrolidone                                                     |
| PYDV               | Potato Yellow Dwarf Virus                                                |
| R                  | Reverse                                                                  |
| rDNA               | Ribosomal DNA                                                            |
| RDV                | Rice Dwarf Virus                                                         |
| R gene             | Resistance gene                                                          |
| RNA                | Ribonucleic Acid                                                         |
| RNA                | RNA interference                                                         |
| RNP                | Ribonucleoprotein                                                        |
| rpm                | Revolutions per minute                                                   |
| RSV                | Rice Stripe Virus                                                        |
| RSMV               | Rice Stripe Mosaic Virus                                                 |

|            |                                                                              |
|------------|------------------------------------------------------------------------------|
| RT         | Reverse Transcriptase                                                        |
| RT-PCR     | Reverse Transcriptase-Polymerase Chain Reaction                              |
| rTtH       | <i>Thermus thermophilus</i>                                                  |
| RubisCo    | Ribulose 1, 5- bisphosphate Carboxylase                                      |
| RYSV       | Rice Yellow Stunt Virus                                                      |
| SbBMV      | Soybean Blotchy Mosaic Virus                                                 |
| SBPH       | Small Brown Plant Hopper                                                     |
| SCV        | Strawberry Crinkle Virus                                                     |
| SDS        | Sodium Dodecyl Sulphate                                                      |
| sec        | Seconds                                                                      |
| siRNA      | Small Interfering RNA                                                        |
| SonV       | Sonchus Virus                                                                |
| SRBSDV     | Southern Rice Black Streaked Dwarf Virus                                     |
| ssDNA      | Single stranded DNA                                                          |
| SSMV       | Sorghum Stunt Mosaic Virus                                                   |
| ss-RT-PCR  | Strand Specific-Reverse Transcriptase-Polymerase Chain Reaction              |
| ss-RT-qPCR | Strand Specific-Reverse Transcriptase-quantitative Polymerase Chain Reaction |
| SYBV       | Soursop Yellow Blotch Virus                                                  |
| SYNV       | Sonchus Yellow Net Virus                                                     |
| SYVV       | Sowthistle Yellow Vein Virus                                                 |
| T          | Thymine                                                                      |
| Taq        | Taq polymerase ( <i>Thermus aquaticus</i> )                                  |
| TaVCV      | Taro Vein Chlorosis Virus                                                    |
| TEM        | Transmission Electron Microscopy                                             |
| Tris-HCl   | Tris-Hydrochloride                                                           |
| TYLCV      | Tomato Yellow Leaf Curl Virus                                                |
| U          | Uracil                                                                       |
| U          | Unit                                                                         |
| UV         | Ultraviolet                                                                  |
| WASMV      | Wheat American Striate Mosaic Virus                                          |
| WCSV       | Wheat Chlorotic Streak Virus                                                 |
| WWRMV      | Winter Wheat Russian Mosaic Virus                                            |
| WYSV       | Wheat Yellow Striate Virus                                                   |
| μl         | Micro litre                                                                  |

$\mu\text{M}$   
%

Micro Molar  
Percentage

## Preface

*Glycine max* (*Fabaceae*, *Phaseoleae*) is a nutritious, annual leguminous plant species native to East Asia. Soybean, or Greater bean as it is sometimes known, was used as a source of protein and oil by the Chinese as early as 2500 B.C (Hartman et al. 1999). Soybean was only introduced into the western world in the 19<sup>th</sup> century, and today sustainable and industrious soybean production is of global importance due to its use for both human consumption and animal feed. In 2016, 742 000 tonnes of soybean was produced on approximately 502 800 hectares in South Africa (faostat.org). However, a number of viral, bacterial, fungal and insect pests and pathogens affect the production of soybean each year. Implementation of control measures aimed at limiting these production losses relies on a clear understanding of the pests and pathogens affecting soybean.

A new disease of soybean, characterized by blotchy mosaic symptoms was identified in the Mpumalanga, North West and Limpopo provinces of South Africa during the early 1990s (Pietersen 1993; Pietersen and Garnett 1990; Pietersen et al. 1998). Examination of viral particle morphology and size by transmission electron microscopy revealed bacilliform-shaped viral particles with a cytoplasmic distribution (Lamprecht et al. 2010). Based on particle morphology, size, intracellular distribution and the sequence of a 522 bp portion of the RNA-dependent RNA polymerase gene the virus was putatively identified as a member of the genus *Cytorhabdovirus* and the name Soybean blotchy mosaic virus (SbBMV) was proposed (Lamprecht et al. 2010).

In order to identify and implement control strategies against SbBMV, a clear understanding of the epidemiology of the virus is required. During previous surveys and studies both the causal agent and insect vector were identified, and information regarding the incidence and distribution of the disease was collected (Lamprecht et al. 2010; Pietersen 1993; Pietersen and Garnett 1990; Pietersen et al. 1998). However, control of the disease in future relies on

the identification of the mechanisms by which the disease persists between soybean growing seasons.

The aim of this study was to investigate the role of alternative reservoir plant hosts, seed transmissibility and mode of vector transmission in the re-introduction of SbBMV onto soybean annually. A positive strand-specific RT-PCR (pss-RT-PCR) was developed to study the mode of transmission of SbBMV in its leafhopper vector, and the diversity present in SbBMV isolates from different temporal and geographical origins was also determined. This thesis is presented as six stand-alone chapters, thus some degree of repetition was unavoidable.

**Chapter 1** entitled “Biology, genomics and vector relationships of plant nucleo- and cytorhabdoviruses” provides an overview of literature on plant nucleo-and cytorhabdovirus biology and transmission. Taxonomy, morphology, replication and genome organization is discussed, and information regarding genetic diversity and virus-insect vector relationships is also provided.

Plants such as weeds and trees which grow in close proximity to crop species often serve as viral reservoirs for plant viruses of crop hosts during the production off-season. Plant viruses that are transmissible through seed also lead to the re-introduction of a virus early in each new production season through spread by insect vectors. **Chapter 2** reports on the identification of alternative plant hosts and evaluation of seed transmissibility of SbBMV in four commercial soybean cultivars commonly grown in areas where high disease incidences are observed annually.

**Chapter 3** describes the development of a positive strand-specific-RT-PCR for SbBMV. This technique allows for the detection of viral replication in plant and insect accessions in which SbBMV is present through RT-PCR amplification of the antigenomic strand, which functions as a replicative intermediate.

A 13 month survey for the leafhopper vector of SbBMV, *Peragallia caboverdensis* is presented in **Chapter 4**. Accessions collected were screened using a diagnostic RT-PCR to determine the proportion of *P. caboverdensis* individuals positive for SbBMV. The pss-RT-PCR developed in Chapter 3 was subsequently employed to investigate the mode of SbBMV transmission by *P. caboverdensis*. The first sequence data for *P. caboverdensis* was also produced with the determination of partial DNA sequences for two genes, mitochondrial cytochrome *c* oxidase subunit I and histone 3.

In **Chapter 5** the amount of genetic diversity present in SbBMV isolates was assessed. Isolates originated from both plant and insect hosts, and were collected over 16 years from seven different regions in the eastern parts of South Africa.

An overview of the results and findings of this thesis is discussed in **Chapter 6**. Final comments on understanding the diversity and mechanisms which facilitate the seasonal persistence of SbBMV in the control of the disease are given, and recommendations for future research are discussed.

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## Research Outputs

### Journal articles

Strydom, E., & Pietersen, G. (2018). Alternative hosts and seed transmissibility of Soybean blotchy mosaic virus. *European Journal of Plant Pathology*, 151(1), 263-268.

Strydom, E., & Pietersen, G. (2018). Diversity of partial RNA-dependent RNA polymerase gene sequences of Soybean blotchy mosaic virus isolates from different host-, geographical- and temporal origins. *Archives of Virology*, 163(5), 1299-1305.

Strydom, E., & Pietersen, G. (2018). Development of a strand-specific RT-PCR to detect the positive sense replicative strand of Soybean blotchy mosaic virus. *Journal of Virological Methods*, 259, 39-44.

### Conference proceedings

Strydom, E & Pietersen, G. Alternative hosts, seed transmissibility and persistent-propagative transmission of Soybean blotchy mosaic virus. *Science Protecting Plant Health Conference*, 26-28 September 2017, Brisbane, Australia (Oral presentation).

Appelgryn, E & Pietersen, G. Alternative hosts and seed transmissibility of Soybean blotchy mosaic virus. *50<sup>th</sup> Anniversary Congress of the South African Society of Plant Pathology*, 15-19 January 2017, Drakensberg, South Africa (Oral presentation).

## Abstract

Infection of soybean by the plant cytorhabdovirus Soybean blotchy mosaic virus (SbBMV) leads to the development of characteristic blotchy mosaic symptoms on leaves. These symptoms re-appear early in each new soybean production season and result in significant yield losses. The persistence of SbBMV between seasons may occur through the presence of reservoir plant hosts during winter, seed transmission or propagation in the leafhopper vector of SbBMV, *Peragallia caboverdensis* as reported for other plant rhabdoviruses. The first aim of this study was to identify alternative hosts of SbBMV in the areas surrounding soybean fields. SbBMV was successfully detected in *Gymnosporia buxifolia*, *Flaveria bidentis* and *Lamium amplexicaule*, and these plant species may serve as viral reservoirs in the absence of soybean. This was followed by a seed transmissibility trial on four commercial soybean cultivars, in which transmission of SbBMV through seed could not be detected. The absence of seed transmissibility and the low frequency of SbBMV mechanical transmission implies that transmission by an insect vector, possibly *P. caboverdensis* is the main method by which SbBMV is spread. The role of *P. caboverdensis* in the dissemination of SbBMV was thus investigated. The year-round presence of *P. caboverdensis* was confirmed at the study site and its association with SbBMV confirmed. *P. caboverdensis* thus has the potential to spread SbBMV between soybean and alternate hosts year-round. A positive strand-specific RT-PCR (pss-RT-PCR) capable of detecting replication of SbBMV was developed to examine the mode of SbBMV transmission by *P. caboverdensis*. No replicative forms of SbBMV, indicative of persistent, propagative transmission of SbBMV, were obtained in any of the leafhopper individuals tested. The first DNA sequence data for *P. caboverdensis* was generated with the sequencing of regions of the mitochondrial cytochrome c oxidase subunit I (*mtCOI*) and histone 3 (*H3*) genes, which will aid species identification without a requirement for morphological expertise. The amount of genetic diversity present in SbBMV populations from different geographical locations, hosts and time of collection was also investigated. Three main lineages, mostly corresponding to geographic origin, were identified. In future, the survey for identification of alternate hosts

should be expanded in order to determine whether additional plant reservoir hosts exists. The establishment of a *P. caboverdensis* colony will allow for the collection of conclusive evidence for the mode of transmission of SbBMV by *P. caboverdensis* by monitoring replication and viral load in insects using the pss-RT-PCR and RT-qPCR respectively. Partial *mtCOI* and *H3* gene sequences should also be determined for additional *P. caboverdensis* accessions from different geographical origins, as well as closely related leafhopper species to confirm accurate species delineation using these two markers. Finally, determination of the whole genome sequence of SbBMV isolates will allow for a phylogenomic approach to determining the evolutionary relationships and genetic diversity present in SbBMV populations. Implementation of chemical control measures such as systemic insecticides, and improved farming and management practices aimed at removing alternate hosts may limit production losses as a result of SbBMV.

# **Chapter 1**

## **Biology, genomics and vector relationships of plant nucleo- and cytorhabdoviruses**

## 1.1 Taxonomy of the family *Rhabdoviridae*

Prior to 2016, rhabdoviruses were classified in the order *Mononegavirales* by the International Committee on Taxonomy of Viruses (ICTV) based on their non-segmented, linear, single-stranded, negative sense RNA genomes and lipoprotein membrane (envelope) (Tordo et al. 2005; Khan and Dijkstra 2006; Dietzgen and Kuzmin 2012). The *Mononegavirales*, established in 1991, initially accommodated the families *Rhabdoviridae*, *Filoviridae* and *Paramyxoviridae*, but to date, eight families have been recognized (*Rhabdoviridae*, *Filoviridae*, *Paramyxoviridae*, *Bornaviridae*, *Nyamiviridae*, *Mymonaviridae*, *Pneumoviridae* and *Suniviridae*) (Amarasinghe et al. 2017). Members are classified in the respective families based on the shape of viral particles, genome complement and organization and mechanisms of gene expression (Jackson et al. 2005; Khan and Dijkstra 2006).

However, in 2016, two genera with bipartite genomes lacking envelopes, the *Dichorhavirus* and *Varicosavirus*, were also included in the family *Rhabdoviridae* (Figure 1.1) (Afonso et al. 2016; Dietzgen et al. 2014; Amarasinghe et al. 2017; Walker et al. 2018). Presently, eighteen genera are designated in the family *Rhabdoviridae* (*Cytorhabdovirus*, *Dichorhavirus*, *Ephemerovirus*, *Lyssavirus*, *Novirhabdovirus*, *Nucleorhabdovirus*, *Perhabdovirus*, *Sigmavirus*, *Sprivivirus*, *Tibrovirus*, *Tupavirus*, *Hapavirus*, *Curiovirus*, *Sripuvirus*, *Ledantevirus*, *Almendravirus*, *Vesiculovirus*, *Varicosavirus*) of which four, the *Nucleorhabdovirus*, *Cytorhabdovirus*, *Dichorhavirus* and *Varicosavirus* infect plants (Amarasinghe et al. 2017; Walker et al. 2018). The different genera were initially distinguished based on serological and antigenic cross-reactivity, but other characteristics such as evolutionary relationships, pathobiology and ecology was also subsequently included (Dietzgen and Kuzmin 2012).

Phylogenetic analysis of conserved motifs in the RNA-dependent RNA polymerase (L) gene of rhabdoviruses has revealed that host (mammal, fish or plant), vector and mode of

transmission shaped the evolution of rhabdoviruses (Bourhy et al. 2005; Kuzmin et al. 2009). Rhabdoviruses infecting fish and plants (novi-, cyto- and nucleorhabdoviruses, dichorhaviruses and varicosaviruses) and which are transmitted by arthropods (cyto- and nucleorhabdoviruses) group separately from rhabdoviruses infecting mammals and reptiles which have dipteran vectors. However, to date the ancestral form of transmission, vector or non-vector transmission, has not been established (Bourhy et al. 2005).

Some species within the *Vesiculovirus*, *Nucleorhabdovirus* and *Cytorhabdovirus* genera also replicate in invertebrates (arthropods), implicating adaptation of rhabdoviruses to a wide range of hosts. The more than 100 members of the family *Rhabdoviridae* described to date thus infect both monocotyledons and dicotyledons plants, invertebrates and animals. This has important implications for agriculture, human and veterinary health, as well as natural ecosystems (Jackson et al. 2005; Hogenhout et al. 2003). Progress in identification of additional characters which can be employed in phylogenetic analysis and the development of new tools will change the way in which scientists approach taxonomy. Future taxonomic analysis of the family will confirm the classifications to date, or suggest different evolutionary relationships within the family (Dietzgen and Kuzmin 2012).

## **1.2 Morphology and particle structure**

With the exception of the dichorha- and varicosaviruses which lack a lipoprotein membrane, members of the family *Rhabdoviridae* have large enveloped particles with a characteristic bullet shaped (animal and human rhabdoviruses) or bacilliform (plant rhabdoviruses) morphology (Figure 1.2A), from which the family derived its name, as *rhabdos* is a Greek word meaning rod shaped (Khan and Dijkstra 2006; Jackson et al. 2005; Kormelink et al. 2011; Dietzgen and Kuzmin 2012; Amarasinghe et al. 2017). Particles vary in length and width, ranging from 100-430 nm to 45-100 nm respectively (Dietzgen and Kuzmin 2012). Due to the distinctive nature of rhabdovirus particle morphology, infection of plant material by members of the family is often confirmed by electron microscopy of plant extracts or of thin

sections of plant material, as viral particles are easily distinguished from cell components (Jackson et al. 2005). Many members of the family have been identified and described only in this manner, and thus require additional molecular characterization to confirm their inclusion in the group (Jackson et al. 2005).

Viral particles consist of a tightly coiled and infectious RNase resistant ribonucleoprotein (RNP) core enveloped by a host-derived lipoprotein membrane (Figure 1.2A) (Jackson et al. 2005; Kuzmin et al. 2009; Khan and Dijkstra 2006; Dietzgen and Kuzmin 2012). The nucleocapsid core is an electron dense structure between 30 nm and 70 nm in diameter, and easily distinguished using electron microscopy (Dietzgen and Kuzmin 2012). The RNP core of most genera comprises a single, linear, negative sense, uncapped and non-polyadenylated genomic RNA with a 5' triphosphate and a 3' free hydroxyl group. The genomic RNA is associated with three viral structural proteins, the nucleocapsid protein (N), the phosphoprotein (P) and a large L protein. The genomic RNA, as well as the antigenomic RNA produced during replication (positive sense RNA) is associated with the N protein permanently, as it is this RNA-N complex which is recognized by the L protein for replication and transcription (Dietzgen and Kuzmin 2012).

The nucleocapsid core is the minimal infectious agent and transcriptionally active (Jackson et al. 2005). The presence of the L protein in virions allows for primary transcription without prior *de novo* host protein synthesis (Dietzgen and Kuzmin 2012). Exclusive processing of the N-RNA complex by the L protein relies on the presence of the P protein which acts as a co-factor (Dietzgen and Kuzmin 2012). This was clearly illustrated in reverse genetics studies in *Sonchus yellow net virus* (SYNV) which showed that co-expression of cloned antigenomic RNAs with plasmids encoding the L, N and P proteins were required to produce infectious SYNV particles (Wang et al. 2015).

The envelope consists of lipoproteins derived from the host (15-25% of virion weight), as well as viral glycoproteins (G proteins), which span the membrane (Jackson et al. 2005;

Dietzgen and Kuzmin 2012). The envelope is attached to the RNP through associations with the matrix protein (M), which has also been postulated to contribute to the coiling of the nucleocapsid core and transcription (Jackson et al. 2005; Dietzgen and Kuzmin 2012). G protein subunits associate as trimers, and these G protein spikes or peplomers are approximately 3 nm in diameter, protruding between 5 nm-10 nm from the viral surface, on which they are arranged as hexamers (Dietzgen and Kuzmin 2012; Jackson et al. 2005).

### **1.3 Plant nucleo- and cytorhabdoviruses**

The plant-adapted rhabdoviruses differ from their human and animal infecting counterparts in two regards. They are able to spread systemically through host plants, which are facilitated by additional movement-associated genes encoded in their genomes, and plant rhabdoviruses are also uniquely adapted to counteracting RNA silencing, an innate immune response of their hosts, plants and insects (Kormelink et al. 2011; Mann et al. 2016). The genera *Nucleorhabdovirus* and *Cytorhabdovirus* are distinguished within the family *Rhabdoviridae* based on the site of replication and accumulation of viral particles in plant cells (Figure 1.3 and Figure 1.4) (Jackson et al. 2005; Dietzgen and Kuzmin 2012). Species are distinguished based on host range and their vectors, and more recently whole genome sequences (Dietzgen and Kuzmin 2012). Crops and weeds in tropical, sub-tropical and temperate regions are infected, with disease symptoms ranging from stunting, clearing and yellowing of veins to mosaic or mottling and necrosis (Jackson et al. 2005).

#### **1.3.1 Genus *Nucleorhabdovirus***

Nucleorhabdoviruses undergo transcription, replication and maturation in the nuclei of plant host cells (Dietzgen and Kuzmin 2012; Jackson et al. 2005). Virions are released through budding from the inner nuclear envelope (INE) into the perinuclear space and membrane-bound cisternae protruding into the cytoplasm from the nucleus (Dietzgen and Kuzmin 2012; Ammar et al. 2005; Jackson et al. 2005). The establishment of viroplasms in the nucleus often results in enlargement of the nucleus. Potato yellow dwarf virus (PYDV) has been

designated the type species of the genus despite the presence of more detailed molecular data for other members such as SYNV and Rice yellow stunt virus (RYSV) (Jackson et al. 2005).

Viral particles gain entry into host cells through feeding of its insect vector on host plants, followed by receptor mediated endocytosis (Khan and Dijkstra 2006; Jackson et al. 2005; Dietzgen and Kuzmin 2012). This is followed by a reduction in the pH of the endosomes, fusion of viral and endosome membranes, and delivery of the RNP into the host cytoplasm (Kuzmin et al. 2009; Jackson et al. 2005). Viral particles are uncoated at the endoplasmic reticulum (ER), and nucleocapsid cores are released. Nuclear pore complexes (NPC) facilitate the targeting of cores to the nucleus, where they become transcriptionally active (Figure 1.3A) (Jackson et al. 2005).

The viral transcriptase transcribes the genomic RNAs repetitively and with a decrease in the abundance of each gene based on its position on the genomic RNA (Banerjee and Barik 1992; Dietzgen and Kuzmin 2012). Genes present closer to the 3' end of the genomic RNA are transcribed at greater levels than those located on the 5' terminus of the genome (N>P>M>G>L) as a result of a proportion of L protein being lost at each gene junction region (Hortamani et al. 2018). The monocistronic mRNAs are capped at their 5' terminus, with a poly-A tail at their 3' ends (Dietzgen and Kuzmin 2012; Jackson et al. 2005). Following transcription in the nucleus, viral mRNAs are exported, and translation of viral mRNAs occurs on ribosomes in the cytoplasm, except for mRNAs encoding the G protein, which are translated on membrane-bound ribosomes. The L protein switches from transcription to replication once sufficient levels of the N protein is reached to encapsidate genomic RNAs (Dietzgen and Kuzmin 2012; Jackson et al. 2005).

Viral proteins are imported into the nucleus, and contribute to replication in viroplasms (Dietzgen and Kuzmin 2012; Jackson et al. 2005). The polymerase transcribes full length positive sense copies of the genome by ignoring transcription stop signals in gene junction

regions. The positive sense genomic RNAs act as replicative intermediates for the synthesis of full length genomic RNAs, secondary transcription and translation. Morphogenesis of viral particles occurs through association of viral nucleocapsids with membrane-bound G proteins and M proteins to facilitate coiling (Dietzgen and Kuzmin 2012; Jackson et al. 2005). Budding occurs through the INE according to the classical model, leading to the expansion of the outer nuclear envelope (ONE). In the alternative model, the INE forms inter-nuclear spherules into which budding occurs, due to proliferation of the INE as a result of cytoplasmic membrane redistribution (Jackson et al. 2005).

### **1.3.2 Genus *Cytorhabdovirus***

In contrast to members of the genus *Nucleorhabdovirus*, cytorhabdoviruses undergo replication and particle assembly in the cytoplasm of plant and insect host cells, similar to that observed for the vertebrate-infecting rhabdoviruses (Kuzmin et al. 2009; Jackson et al. 2005; Dietzgen and Kuzmin 2012). Cytorhabdoviruses are released through budding into vesicles of the ER and associated cytoplasmic cisternae where virions accumulate (Dietzgen and Kuzmin 2012). Lettuce necrotic yellows virus (LNYV) is considered the type species of the genus *Cytorhabdovirus*, and has been characterized in the most detail (Tordo et al. 2005; Jackson et al. 2005).

Feeding of insect vectors through their stylus mediates entry into plant hosts. Uncoating of viral particles occurs at the ER, and nucleocapsid cores are released into the cytoplasm (Jackson et al. 2005; Dietzgen and Kuzmin 2012). The cores associate with the ER to form viroplasms, where viral mRNAs are transcribed and copies of both genomic and antigenomic RNAs are produced (Figure 1.3B). After translation of viral mRNAs, proteins involved in replication accumulate in viroplasms, while G proteins amass at the cytoplasmic membranes or the ONE (Jackson et al. 2005). Maturation of viral particles take place at sites of G protein accumulation through M protein mediated condensation of cores, and viral particles are

released by budding into the ER with its associated viroplasms (Jackson et al. 2005; Dietzgen and Kuzmin 2012).

### **1.3.3 Soybean blotchy mosaic virus (SbBMV)**

A survey for the identification of viruses in soybean (*Glycine max*) production areas in the North West, Limpopo and Mpumalanga provinces of South Africa in the early 1990's identified a new virus-like disease, characterized by blotchy mosaic symptoms (Pietersen 1993). Symptoms became prevalent early in the growing season, declining with time. A strong correlation between disease symptoms and rhabdovirus-like particles was observed, and disease incidence ranged between 0% and 32%. This related to yield losses of between 0% and 20%, depending on the cultivar grown in the warmer regions at lower altitudes, where the disease was prevalent (Pietersen 1993).

During a follow-up survey conducted in the 1993/1994 and 1994/1995 soybean growing seasons, the disease was even more prevalent (Pietersen et al. 1998). Disease incidence was high in the Brits/Thabazimbi, Loskopdam Irrigation Scheme, Lydenburg/Origstad, Machadodorp/Badplaas, and Greytown/Weenen areas. The virus was detected in 20.9% of the 695 samples collected, and disease incidence ranged between 10%-50% in a field (Pietersen et al. 1998). To date, the disease has been detected in the North West, Gauteng, Limpopo, Mpumalanga, KwaZulu-Natal and Eastern Cape provinces of South Africa (Strydom and Pietersen 2018; Pietersen and Garnett 1990; Pietersen et al. 1998).

Subsequent isolation and partial characterization of the virus indicated that it was a putative member of the genus *Cytorhabdovirus* (Lamprecht et al. 2010). Virus particles ranged between 338 to 371 nm by 93 nm in size, and were bullet-shaped, with a cytoplasmic distribution (Lamprecht et al. 2010). The virus was successfully transmitted by mechanical transmission, and a leafhopper, *Peragallia caboverdensis* Lindberg (*Cicadellidae: Megophthalminae: Agalliini*) was identified as its vector. The name Soybean blotchy mosaic virus (SbBMV) was proposed (Lamprecht et al. 2010).

## 1.4 Genome organization

To date, the whole genome sequence of 25 plant rhabdoviruses has been determined (Table 1.1). However, 11 of the species for which whole genome sequences are available on the National Centre for Biotechnology Information (NCBI) database has not been accepted as classified to species level by the ICTV. Whole genome sequences are available for the ICTV-recognized cytorhabdovirus species LNYV (Wetzel et al. 1994a; Dietzgen et al. 2006; Wetzel et al. 1994b), Northern cereal mosaic virus (NCMV) (Tanno et al. 2000), Lettuce yellow mottle virus (LYMoV) (Heim et al. 2008), Alfalfa dwarf virus (ADV) (Bejerman et al. 2015), Strawberry crinkle virus (SCV) (Koloniuk et al. 2018), Barley yellow striate mosaic virus (BYSMV) (Yan et al. 2015), Wheat yellow striate virus (WYSV) (Liu et al. 2018) and Colocasia bobone disease-associated virus (Higgins et al. 2016a). Persimmon virus A (Ito et al. 2013), Cabbage cytorhabdovirus 1 (Pecman et al. 2017), Wuhan insect viruses 4, 5 and 6 (Li et al. 2015), Maize yellow striate virus (MYSV) (Maurino et al. 2018), Rice stripe mosaic virus (RSMV) (Yang et al. 2017), Maize-associated rhabdovirus (Willie and Stewart 2017) and Yerba mate rhabdovirus (Bejerman et al. 2017) still need to be classified to species level.

Genome sequences for nine species within the genus *Nucleorhabdovirus* have been completed. These are PYDV (Ghosh et al. 2008; Jang et al. 2017), Maize fine streak virus (MFSV) (Tsai et al. 2005), Maize mosaic virus (MMV) (Reed et al. 2005), RYSV (Huang et al. 2003), SYNIV (Heaton et al. 1989; Choi et al. 1994; Zuidema et al. 1986; Scholthof et al. 1994), Iranian maize mosaic virus (IMMV) (Massah et al. 2008; Ghorbani et al. 2018), Eggplant mottled dwarf virus (EMDV) (Babaie et al. 2015; Zhai and Pappu 2014), Datura yellow vein virus (DYVV) (Dietzgen et al. 2015) and Taro vein chlorosis virus (TaVVCV) (Revill et al. 2005), Blackcurrant nucleorhabdovirus 1 (Wu et al. 2018) and Physostegia chlorotic mottle virus (Gaafar et al. 2018; Menzel et al. 2018). Blackcurrant nucleorhabdovirus 1 and Physostegia chlorotic mottle virus have been provisionally classified as nucleorhabdoviruses.

Genome sizes range from 12 kb to 14.5 kb, and contain six to nine open reading frames (ORFs), flanked by a 3' leader sequence and a 5' trailer sequence (3'-leader-N, P, M, G, L-trailer-5'), and genes are separated by short spacer sequences (Figure 1.5) (Jackson et al. 2005; Dietzgen and Kuzmin 2012). The six ORFs encode five conserved structural proteins present in all plant rhabdoviruses, and one or more accessory genes. Leader sequences in plant rhabdoviruses range in size from 84 nt to 206 nt, while trailer sequences are usually between 145 nt and 389 nt in length. Leader and trailer sequences differ inter- and intraspecifically, but often possess small regions of complementarity at the termini. These sequences contain transcription and replication initiation signals (promoters) to initiate replication of the genome and antigenome (Dietzgen and Kuzmin 2012; Jackson et al. 2005).

Gene junction regions are highly conserved between the different genes of a virus and among plant rhabdoviruses, and have been postulated to function in the regulation of transcription and replication (Jackson et al. 2005; Dietzgen and Kuzmin 2012). This is in contrast to the leader, trailer and protein coding sequences, which show considerable sequence variation, probably in order to allow adaptation to different environments. Intergenic regions are divided into three components or elements, known as element I, element II and element III (Dietzgen and Kuzmin 2012; Jackson et al. 2005).

Element I comprises the polyadenylation signal consisting of a 8-9 nucleotide poly (U) tract interspersed with A or C nucleotides at the 3' sequence end of each gene (Reed et al. 2005). Element II is a short non-transcribed sequence that separates each gene, often containing G nucleotides, and element III a conserved sequence functioning as the transcriptional start site found at the beginning of each new gene (Jackson et al. 2005). For most rhabdoviruses such as Rabies virus, Vesicular stomatitis virus and the nucleorhabdoviruses, the codon UUG signals initiation of transcription. Conversely, the cytorhabdoviruses NCMV and LNYV use the codons CUA and CUU to initiate transcription respectively (Reed et al. 2005; Jackson et al. 2005).

The genomes of all sequenced plant rhabdoviruses contain between one and four additional ORFs at position X between the *P* and *M* genes (Figure 1.5) (Jackson et al. 2005; Khan and Dijkstra 2006; Dietzgen and Kuzmin 2012; Walker et al. 2011). The exception is PYDV, which encodes an ancillary protein in a gene positioned between the *N* and *P* genes (Bandyopadhyay et al. 2010). These ORFs are mostly encoded between existing ORFs in plant rhabdoviruses, but may have overlapping ORFs in some plant rhabdoviruses and other groups of rhabdoviruses such as the vesiculoviruses and ephemeroviruses (Dietzgen and Kuzmin 2012; Walker et al. 2011). The 4b (LNYV) (Dietzgen et al. 2006; Wetzel et al. 1994a), sc4 (SYNV) (Scholthof et al. 1994), P3 (RYSV, SCV, MMV, ADV, DYVV, IMMV, TaVVCV, WYSV) (Huang et al. 2005; Bejerman et al. 2015; Dietzgen et al. 2015; Reed et al. 2005; Massah et al. 2008; Schoen et al. 2004; Revill et al. 2005; Liu et al. 2018), Y (PYDV) (Bandyopadhyay et al. 2010) and P3 and P4 (MFSV) proteins are all encoded by ORFs at position X and are postulated to be involved in cell-to-cell movement (Dietzgen et al. 2006; Jackson et al. 2005; Dietzgen and Kuzmin 2012). BYSV and NCMV contain three and four accessory genes at this position respectively (Yan et al. 2015; Tanno et al. 2000), with two of the genes encoded in alternate frames within the middle transcriptional unit in BYSV.

The secondary structures of these proteins resemble that of viral movement proteins in the 30K superfamily, and bind both single-stranded RNAs and N proteins, the predominant protein in RNPs (Huang et al. 2005; Dietzgen and Kuzmin 2012; Jackson et al. 2005; Walker et al. 2011). The sc4 protein of SYNV associates with membranes and cell walls (Scholthof et al. 1994), and the RYSV P3 protein is able to complement a movement-deficient potexvirus in *Nicotiana benthamiana* (Huang et al. 2005). Similarly, deletion analysis of the sc4 gene in SYNV using a reverse genetics system illustrated the requirement of the sc4 gene for movement of nucleocapsid cores between cells (Wang et al. 2015). During the intermediate stages of infection, viral nucleocapsids associate with virus-encoded movement proteins to facilitate the spread of viral particles to neighbouring cells. These proteins are proposed to have a role in the binding of nucleocapsids, transport through nuclear pore

complexes to the plasmodesmata, and exclusion limits of the plasmodesmata (Jackson et al. 2005).

In some plant rhabdoviruses, such as SCV, WYSV (P6) (Schoen et al. 2004; Liu et al. 2018), ADV (P6) (Bejerman et al. 2015), NCMV (P9), BYSMV (P9) (Yan et al. 2015) and RYSV (P6) (Huang et al. 2003), additional genes are also present at position Y between the *G* and *L* genes. The highly acidic P6 protein at this position in RYSV has been shown to contribute to virulence by acting as a suppressor of virus-induced gene silencing in plant hosts (Guo et al. 2013). However, all other proteins encoded by accessory genes at position Y are basic, with no sequence homology between genes (Jackson et al. 2005; Walker et al. 2011).

Differentiation between nucleo- and cytorhabdoviruses based on gene complements is not possible as a result of inconsistent genome organization within the genera (Figure 1.5) (Dietzgen and Kuzmin 2012; Jackson et al. 2005, Walker et al. 2011). RYSV encodes an accessory protein, P6 (Huang et al. 2003), between the *G* and *L* genes, which is also present in the cytorhabdoviruses BYSMV (Yan et al. 2015) and NCMV (Tanno et al. 2000). MFSV in turn encodes two putative movement proteins between the *P* and *M* genes, while only one is present in RYSV, MMV, SYNV, PYDV and IMMV. Additionally, the genome organization of the cytorhabdoviruses LNYV (Dietzgen et al. 2006) and LYMoV (Heim et al. 2008) are identical to that of the nucleorhabdovirus SYNV. No consistency in the number and position of accessory genes is thus found in the nucleo- and cytorhabdoviruses.

### **1.5 Phylogenetic relationships and genetic diversity**

Conserved portions of the *L* gene corresponding to the RNA-binding and polymerase motifs, and regions of the *N* gene are widely used to infer phylogenetic relationships within the *Rhabdoviridae* and its respective genera, and to assess the genetic diversity present in different isolates of a species (Kuzmin et al. 2009; Jackson et al. 2005; Bourhy et al. 2005; Klerks et al. 2004; Revill et al. 2005; Callaghan and Dietzgen 2005; Talbi et al. 2011; Petrzik 2012). Phylogenies based on *L* and *N* gene sequence data suggest a monophyletic origin

for the plant rhabdoviruses (Bourhy et al. 2005; Kuzmin et al. 2009). In these phylogenies, nucleo- and cytorhabdoviruses separate into two distinct lineages, with two closely related sister clades present in the genus *Nucleorhabdovirus* (Hogenhout et al. 2003; Kuzmin et al. 2009; Bourhy et al. 2005). However, as the complete genomes of more plant rhabdoviruses become available, whole genome sequences (phylogenomics) will replace the partial sequences of a few gene regions in investigating the evolutionary relationships and diversity of viral species and isolates.

The phylogenetic relationships and genetic diversity of a number of plant nucleo- and cytorhabdoviruses has been investigated. Phylogenetic analysis of a region encoding the GDN-motif of the *L* gene of SCV revealed the presence of two distinct groups of SCV isolates among eight European field isolates (Klerks et al. 2004). The partial *L* gene sequence was 98% similar within the subgroups, with an 11% difference between groups. No relationships between the grouping of isolates and geographical location or symptom severity were observed (Klerks et al. 2004).

Analysis of the complete gene sequence of the *N* gene in eight LNYV isolates collected between 1985 and 2003 also revealed the presence of two distinct subgroups in Australia. As reported for SCV, no correlation was found between these groups and separation in both space and time (Callaghan and Dietzgen 2005). Nucleotide sequences were 96% similar for isolates within the same subgroup, and differed by 20% between subgroups. The 20% difference in nucleotide similarity only translated into a 4% difference in amino acid sequence, revealing a preference for synonymous mutations at the third codon position (Callaghan and Dietzgen 2005). In a subsequent study, however, sequences corresponding to the *N* gene of isolates from both Australia and New Zealand were analysed, and isolates grouped in two distinct clades based on date of isolation (Higgins et al. 2016b).

In contrast to the two examples discussed above, phylogenetic analysis of approximately 1kb of both *N* and *L* gene sequence data from TaVCV resulted in the formation of distinct

subgroups correlated to geographical location in the Pacific Islands in most cases (Revill et al. 2005). Percentage differences in nucleotide and amino acid sequences were 27.4 and 19.3, and 11.3 and 6.3 for the *L* and *N* genes respectively (Revill et al. 2005). Studies based on sequence data generated for N, X, P, Y, M, G and UTRs of EMDV also mostly resulted in the formation of subgroups based on geographical origin of isolates in Greece and Cyprus (Pappi et al. 2016). Low levels of genetic diversity was found in all gene regions analysed except for X and the UTRs. EMDV isolates from Italy, Greece and Spain also separate into distinct clades according to geographic origin upon analysis of partial *L* gene sequences (Parrella and Greco 2016). Grouping according to geographic origin, symptom severity or date of isolation for plant rhabdoviruses in which evolutionary relationships have been studied to date thus does not appear to be conserved.

## **1.6 Ecological and epidemiological factors affecting the spread of plant nucleo- and cytorhabdoviruses**

As a result of the obligate dependence of viruses on their hosts for replication and on their vectors for spread, plant virus hosts and insect vectors play an important role in shaping the geographical and host range, incidence and persistence of plant viruses (Wilson 2014). The availability of a susceptible plant host coupled with spread through a competent insect vector allows for the establishment of disease. However, in order for disease to persist in periods where the host plant is absent, alternative plant hosts and prolonged associations with insect vectors or plant propagules are required.

### **1.6.1 Insect vectors of plant nucleo- and cytorhabdoviruses**

Most rhabdoviruses are transmitted by insect vectors, but some groups such as the lyssaviruses and novirhabdoviruses have evolved the ability to circulate in vertebrates without the requirement for arthropod vectors (Dietzgen and Kuzmin 2012). Plant rhabdoviruses are commonly transmitted by insects with sucking mouthparts such as aphids (*Aphidoidea*), leafhoppers (*Cicadellidae*) and planthoppers (*Fulgoroidea*) (Khan and Dijkstra

2006; Dietzgen and Kuzmin 2012; Blanc et al. 2014; Jackson et al. 2005), but reports of lacebugs (*Piesmididae*) and brevipalpus mites (*Acarina*) (Kitajima et al. 2010) serving as vectors for putative plant rhabdoviruses are also present in literature.

### **1.6.2 Modes of transmission**

The transmission of plant viruses by members of the *Hemiptera* (aphids, whiteflies, planthoppers and leafhoppers) can be described as non-persistent, semi-persistent, persistent, circulative or persistent and propagative (Nault 1997; Nault and Rodriguez 1985). Additionally, the terms “stylet-borne” or “foregut-borne” are often added to describe non-persistent and semi-persistent virus transmission respectively, depicting the sites of virus accumulation in vectors. The mode of transmission and persistence of viruses appears to be conserved within genera (Nault 1997).

Non-persistent and semi-persistent transmission of plant viruses by arthropod vectors is characterized by the absence of a latent period in the host, loss of infectivity after a few minutes or hours, and also following moulting (ecdysis) (Nault 1997; Nault and Rodriguez 1985). Virions are located in the mouth of the insect (non-persistently transmitted viruses) and the foregut (semi-persistently transmitted viruses), which is shed during moulting. Plant viruses transmitted non-persistently are acquired in seconds or minutes upon feeding, and only retained for a few minutes. During semi-persistent transmission, a few short (minutes or hours) feedings are sufficient for acquisition and inoculation, and insects remain infective for longer time periods upon prolonged feeding on infected plant material (Nault and Rodriguez 1985; Nault 1997).

The majority of hopper-borne viruses are transmitted persistently (Nault and Rodriguez 1985; Nault 1997). Persistent or circulative transmission can be either propagative or non-propagative, and higher transmission efficiencies are reached when the virus is acquired by nymphs (Nault 1997; Nault and Rodriguez 1985). During propagative transmission, the virus replicates within the vector, while it only circulates in the host during non-propagative

transmission. Feeding for hours or days is required for acquisition of the virus, and the vectors remain viruliferous for days or weeks in the case of persistent, circulative viruses, and weeks and months for viruses transmitted in a persistent, propagative manner (Nault and Rodriguez 1985).

It is hypothesized that persistent and non-persistent plant viruses alter host plants in such a manner to optimize their feeding strategy (Mauck et al. 2012). While both persistently and non-persistently transmitted viruses make the plant host more attractive to vectors, infection with persistently transmitted viruses often leads to an increase in host quality for long term feeding. Infection of a host plant by a non-persistent virus generally results in a reduction in host quality, promoting rapid vector dispersal (Mauck et al. 2012).

#### **1.6.2.1 Persistent, propagative transmission of plant rhabdoviruses**

Replication of plant rhabdoviruses in their hemipteran vectors has been reported in all virus-vector associations in which transmission was studied, implying that plant rhabdoviruses have two natural hosts, both the plants they infect as well as their insect vectors (Hogenhout et al. 2003; Dietzgen and Kuzmin 2012; Nault 1997). Long latent periods between acquisition and transmission, occurrence throughout the life cycle of the vector and transovarial transmission serve as indirect evidence for the replication of plant rhabdoviruses in their vectors (Dietzgen and Kuzmin 2012; Nault 1997; Jackson et al. 2005). Transmission following serial dilution passages, electron microscopy, serological detection, infection of specific insect tissue culture lines by specific virus strains and monitoring of virus titre can be used for more direct confirmation of replication (Jackson et al. 2005; Nault and Rodriguez 1985; Nault 1997). Replication in their respective vectors has been shown by various different approaches such as microscopy, serological assays and transcriptomic and RT-qPCR studies for the plant rhabdoviruses IMMV, MMV, MFSV, NCMV, BYSV, Wheat chlorotic streak virus (WCSV) and RYSV (Ammar and Hogenhout 2008; Barandoc-Alviar et

al. 2016; Whitfield et al. 2015; Conti 1985; Ammar and Nault 1985; Falk and Tsai 1985; Todd et al. 2010; Massah et al. 2008).

The integration of rhabdovirus genes into the genomes of several arthropod species has been reported, which supports the close evolutionary relationships described between rhabdoviruses and their invertebrate hosts (Fort et al. 2012). Plant viruses such as the plant rhabdoviruses which are capable of replicating in their insect vectors thus most probably originated from insects, and a host jump led to their adaptation to plants (Matthews 1991). The presence of the virus in the plant host can often lead to the development of disease, especially in newly-introduced crop species, while disease does not occur in insect vectors.

Plant viruses with hemipteran vectors are typically transmitted by species of one family or families from the same sub-order (Nault 1997; Dietzgen and Kuzmin 2012; Nault and Rodriguez 1985). Plant rhabdoviruses have very specific interactions with their insect vectors, and more often than not only one or a few closely-related species transmit a virus. *Endria inimica* is not able to transmit MFSV (Redinbaugh et al. 2002), while *Graminella nigrifrons*, the vector of MFSV, is able to. Correspondingly, *G. sonora* and not *G. nigrifrons* is able to transmit Sorghum stunt mosaic virus (SSMV) (Creamer et al. 1997).

Nevertheless, when more than one vector exists, specific species act as the primary vector, transmitting the virus more effectively than others. As an example, the transmission efficiency of RYSV by *Nephotettix virescens* is lower than what is observed for *N. cincticeps* and *N. nigropictus* (Hibino 1996), and *E. inimica* transmits Wheat american striate mosaic virus (WASMV) more efficiently than *Elymana virescens* (Sinha 1970). *N. cincticeps*, *N. nigropictus* and *E. inimica* are thus more competent vectors of RYSV and WASMV respectively than *N. virescens* and *E. virescens*.

Vector competence is defined as the percentage of a vector population that can transmit a virus to new plant hosts (Dietzgen and Kuzmin 2012). Factors such as different vector populations, sizes, sexes and developmental stages can affect vector competence

(Sylvester and Richardson 1992; Todd et al. 2010). MFSV accumulates to higher levels in nymphs than in adults (Todd et al. 2010), and there is variation among individuals within reared populations of *Peregrinus maidis* and *E. inimica* in the abilities to transmit MMV and WASMV, respectively (Slykhuis 1963; Ammar et al. 1987).

Cyto- and nucleorhabdoviruses do not have an association with a specific family of vectors such as the leafhoppers or planthoppers. The cytorhabdoviruses BYSMV, Festuca leaf streak virus (FLSV) and NCMV, as well as the nucleorhabdoviruses MMV, Cynodon chlorotic streak virus (CCSV) and IMMV are transmitted by planthoppers (*Delphacidae*) (Dietzgen and Kuzmin 2012). Similarly, both nucleorhabdoviruses SSMV, RYSV, MFSV and Cereal chlorotic mottle virus (CCoMV) and cytorhabdoviruses WASMV and Winter wheat russian mosaic virus (WWRMV) are transmitted by leafhoppers (*Cicadellidae*) (Dietzgen and Kuzmin 2012; Jackson et al. 2005).

As a result of the close relationship between plant rhabdoviruses and their insect vectors, the disease incidence, distribution and host range of plant rhabdoviruses is often delimited by the occurrence of the vector (Jackson et al. 2005; Dietzgen and Kuzmin 2012). As an example, the incidence of SYVV drastically declined in California due to the introduction and resulting competitive exclusion of its aphid host *Hyperomyzus lactucae* by an invasive aphid species *Uroleucon sonchi* (Sylvester and Richardson 1992).

#### **1.6.2.2 Horizontal and vertical transmission of plant nucleo- and cytorhabdoviruses in insect vectors**

The process by which insect vectors spread plant viruses is divided into three phases: acquisition, the latent period and inoculation (Nault and Rodriguez 1985; Nault 1997). The insect vector acquires the plant virus during feeding on infected leaves, and virions have to overcome numerous physical barriers in the midgut and salivary glands in order to reach the salivary glands for further horizontal spread (Figure 1.6). During their systematic movement throughout the body of the insect vector virions also have to avoid and modulate the insect's

innate immune response and its associated biochemical pathways (Jia et al. 2018; Whitfield et al. 2015)

During acquisition, insects obtain the virus by feeding on plant material containing the virus, or alternatively, congenitally by transovarial passage from their infected mothers (Jia et al. 2018; Nault and Rodriguez 1985). At this stage, the insect vector is viruliferous, but not competent. Acquisition times of plant rhabdoviruses have been shown to range from 1-15 minutes depending on their distribution in the tissues of their plant hosts (mesophyll or phloem), but longer feedings increase the number of individuals which acquire the virus (Nault 1997; Jia et al. 2018; Nault and Rodriguez 1985). WASMV, which infects both mesophyll and phloem cells can be acquired within one minute, while RYSV, a phloem-limited nucleorhabdovirus, requires 15 min (Slykhuis 1963; Lee 1967; Chiu et al. 1968; Inoue 1979). Other factors besides virus acquisition time can also determine acquisition efficiency. During feeding, insects can release effector-like molecules which activate the host plant's defence responses, lowering acquisition efficiency (Hogenhout and Bos 2011).

A transcriptomic study of MMV-infected *P. maidis* guts identified expressed sequence tags (ESTs) with high amino acid similarity to known rhabdovirus receptors and endocytosis-related proteins in mammals (Whitfield et al. 2011). Plant rhabdoviruses thus utilize receptor-mediated endocytosis pathways similar to those found in mammals to mediate the entry of viral particles into the midgut of insects. Interactions between G protein spikes on the viral surface with receptors situated on the insect cell surface allows for entry into the gut of the vector. These receptors in the gut and salivary glands of insects are major determinants of host-specificity and competency (Blanc et al. 2014).

Vertical transmission of plant rhabdoviruses has only been reported for a few virus species, predominately those transmitted by aphids (Dietzgen and Kuzmin 2012; Sinha 1970). The mechanisms by which vertical transmission occurs is largely unknown, but it is believed that viruses exploit existing pathways associated with movement of vector proteins either directly

or through virus-induced tubules (Jia et al. 2018). Transport to the oocytes can occur through binding to vector host proteins such as yolk protein precursors, which are targeted to the oocyte or associated with virus-induced tubules by microvilli from follicular cells into the oocytes (Jia et al. 2018; Liao et al. 2017).

Another mechanism of entry into oocytes relies on the association of virions to obligate symbiont bacteria of leafhoppers (Jia et al. 2018). A direct association between Rice dwarf virus (RDV) and *Sulcia* spp, an obligate pathogen of the rice green leafhopper *N. cincticeps* has been observed (Jia et al. 2017). Paternal transmission has also been described in bacterial and insect viruses, but evidence of plant viruses utilizing this mechanism for vertical transmission has not been found to date. The symbiotic rhabdovirus of *Drosophila*, Sigma virus, has however been shown to be transmitted through infected males (Longdon et al. 2011; Longdon et al. 2017; Watanabe et al. 2014), thus investigation into possible interactions between plant rhabdovirus G proteins and proteins on the surface of sperm cells will be interesting in future. Vertical transmission may play an important role in the seasonal persistence of especially crop-specific viruses when plant viral reservoirs might be absent (Jia et al. 2018).

The latent period, which is absent during semi-persistent virus transmission, is defined as the time period required for viruliferous insects to become infective (Dietzgen and Kuzmin 2012). This involves viral particles moving from the gut to the salivary glands of insect vectors (Nault and Rodriguez 1985; Nault 1997; Dietzgen and Kuzmin 2012). The latent period of propagative viruses is often similar to the incubation time of host plants, and is generally specific for every virus-vector relationship. Latent periods of plant rhabdoviruses are also shorter in competent versus incompetent vectors, and can range widely in a specific virus-vector interaction (Slykhuis 1963). *Nephotettix* spp., the vectors of RYSV require between 3 and 66 days in order to become infective, while MFSV's vector, *G. nigrifrons*, requires at least 28 days (Chiu et al. 1968; Todd et al. 2010).

During the latent period, infection foci are established in the midgut epithelium of the insect, after which virions move to midgut visceral muscles (Jia et al. 2018; Whitfield et al. 2015; Dietzgen and Kuzmin 2012). Persistent, non-propagative viruses such as the luteo- and begomoviruses cross midgut barriers as intact virions (Brault et al. 2007; Medina et al. 2006), while persistent, propagative viruses use viral inclusions (Jia et al. 2014; Chen et al. 2012). This process has been widely studied in rice reoviruses, in which viral non-structural proteins form tubular structures which they use to pass through the microvilli and the basal lamina from the midgut epithelium into the lumen, and visceral muscles of the vector respectively (Figure 1.6B) (Whitfield et al. 2015). These tubules involved in actin-based tubule motility are powered through membrane and tissue barriers by the midgut actin-myosin complex (Chen et al. 2017). The role of the midgut barrier in restricting viral movement is illustrated most vividly in experiments on *P. maidis* in which MMV was injected into the thorax, which led to increased numbers of infected individuals (Falk and Tsai 1985).

Persistently transmitted viruses move from the midgut to the salivary glands through the hemolymph or direct interactions with other cellular structures (Figure 6) (Whitfield et al. 2015; Jia et al. 2018; Dietzgen and Kuzmin 2012). In the begomo- and luteoviruses, virions interact with endosymbiotic bacteria in the hemolymph to gain entry into the salivary glands (Gottlieb et al. 2010; van den Heuvel et al. 1997). Reoviruses utilize virus-induced filaments to mediate interactions between viral proteins and host actin proteins, which triggers crossing of the plasmalemma barrier through exocytosis, facilitating entry into the salivary glands (Figure 6C) (Mao et al. 2017). In the interaction between the Rice stripe virus (RSV) and the small brown plant hopper (SBPH), the RSV non-structural protein NS4 facilitates the spread of RSV in the vector through a direct interaction with the nucleoprotein (Wu et al. 2014). Knock-down of this protein through RNAi significantly affects the spread, and not the replication of the virus. Some plant rhabdoviruses such as FLSV, MMV and MFSV can also follow a different route to the salivary glands via the vector nervous system (Ammar and Hogenhout 2008; Todd et al. 2010; Lundsgaard 2018).

Virions move from the secretory region to the salivary cavities, from where it passes through the salivary canal into the plant (Jia et al. 2018; Dietzgen and Kuzmin 2012). MMV buds through the plasma membranes of the secretory cells and collects near secretory vesicles in inter- and intracellular spaces for secretion into saliva (Ammar et al. 1985). The inability of a virus to cross barriers associated with the salivary glands implies that it can infect and replicate within an insect host, without being transmitted, with the insect subsequently classified as a non-vector (Dietzgen and Kuzmin 2012). The number of individuals in a population in which the virus is present in the salivary glands is usually lower than the number of individuals harbouring the virus in the midgut, highlighting the importance of the physical barriers associated with entry into the salivary glands. Once virions reach the salivary glands, insects are mostly inoculative (Todd et al. 2010; Ammar and Hogenhout 2008).

Other factors affecting vector competence besides physical anatomical barriers include insect anti-viral defence mechanisms such as siRNA and autophagy pathways (Dietzgen and Kuzmin 2012; Jia et al. 2018). Recognition of Southern rice black streaked dwarf virus (SRBSDV) by siRNA defence pathways in its non-competent vector, the SBPH prevents accumulation and spread of the virus in its insect vector (Lan et al. 2016). In the whitefly *Bremisia tabaci* infection by the begomovirus Tomato yellow leaf curl virus (TYLCV) results in the activation of *B. tabaci*'s autophagy pathway, leading to the degradation of the TYLCV coat protein and DNA (Wang et al. 2016). Similarly, expression of 202 EST's corresponding to genes involved in insect innate immunity was identified in the gut of *P. maidis* infected with MMV (Whitfield et al. 2011). Insects can thus, like plants, activate a suite of biochemical pathways in response to viral attack in order to limit the spread of the virus.

The final stage, inoculation, involves the introduction of viral particles into uninfected plant material through feeding (Nault and Rodriguez 1985; Nault 1997). Rate of transmission from the insect to the plant increases with increased feeding time (Dietzgen and Kuzmin 2012; Nault 1997; Nault and Rodriguez 1985). The number of viruliferous insects in a population is

often greater than the number of infective insects, as all insects which acquire the virus do not become infective as a result of physical or biochemical barriers in the vector (Dietzgen and Kuzmin 2012). Literature reports that MFSV and MMV have been detected at levels of 30-60% and 28% in their vectors *G. nigrifrons* and *P. maidis*, but that only 10% and 17% of individuals were inoculative respectively (Ammar and Hogenhout 2008; Todd et al. 2010).

Viruses also possess mechanisms to modulate insect defence responses. The outer capsid protein of RSV can promote its own proliferation through activating the c-Jun N terminal kinase pathway in the SBPH (Wang et al. 2017). A vector protein then further associates with the outer capsid protein, protecting the viral particles from the insect immune system (Liu et al. 2015). Correspondingly, the P protein of LNYV has been shown to act as a suppressor of RNA silencing (Mann et al. 2015), protecting LNYV from virus-induced insect immune responses. Virus proteins thus act in a similar manner to fungal and bacterial effectors to manipulate the insect's immune responses, and an equilibrium between these two opposing forces is reached to allow for the long term persistence of propagative viruses in their insect vectors.

#### **1.6.2.3 *Peragallia caboverdensis*, a leafhopper vector of SbBMV**

With more than 20 000 spp. representing approximately 2400 genera, the leafhoppers are the largest family of sap-sucking insects and insect vectors (Dietrich 2013). Leafhoppers are economically important in agriculture as they can be pests in themselves or transmit plant pathogens. Leafhoppers exhibit a global distribution which includes extremes such as tropical rainforests and the arctic, and feed on the phloem of all groups of vascular plants, and are often considered an indicator species of the health of habitats (Hollier et al. 2005; Nickela and Hildebrandt 2003). The taxonomy, phylogeny and ecology of the leafhoppers still remain largely unknown, but it is clear that they evolved closely with their plant hosts and parasites (Degman et al. 2011; Saur-Kruh et al. 2013; Moran et al. 2005; Li et al. 2015).

The leafhopper *P. caboverdensis* has been proposed as the insect vector of SbBMV (Lamprecht et al. 2010). Individuals of *P. caboverdensis* were capable of transmitting SbBMV to healthy soybean plants after feeding on SbBMV-infected unrooted cuttings for 7 days (Lamprecht et al. 2010). The characteristic blotchy mosaic symptoms developed within two months, and rhabdovirus particles were detected using immunoelectron microscopy (IEM). SbBMV could only be transmitted to soybean through two serial passages, after which individuals of *P. caboverdensis* were no longer viruliferous (Lamprecht et al. 2010).

Little information is available for *P. caboverdensis*, which was first described by Lindberg from the Cape Verde Islands (Lindberg 1985). *P. caboverdensis* was subsequently also reported from Salvages, Aldabra Islands, Morocco, Mauritius, Madeira and Rhodesia (Zimbabwe) (Lindberg 1965; Webb 1980; Quartau and Andre 1980). *P. caboverdensis* was hypothesized to be endemic in the Cape Verde Islands by Lindberg, but a wide distribution in Africa has subsequently been proved.

Males as well as females are a whitish brown, with females slightly larger than males (average length 3.4 mm vs 3.09 mm). Both males and females have two roundish spots on both the crown and the pronotum, with the spots larger on the pronotum in males. During the expedition to the Salvages (islands in the North Atlantic), *P. caboverdensis* was found colonizing *Lycopersicon esculentum* with another leafhopper, *Brachipteronia vieirai* (Quartau and Andre 1980).

In future, SbBMV transmission parameters such as optimal acquisition and transmission times, optimal temperatures and latent periods should be established. Possible persistent, propagative transmission of SbBMV in *P. caboverdensis*, as reported for other plant rhabdoviruses should be investigated, and the presence of additional insect vectors evaluated.

### 1.6.3 The role of alternative plant hosts

Volunteer plants, wild plant species, weeds and alternate and overlapping crops play an important role in the ecology and epidemiology of plant viruses (Wilson 2014; Wisler and Norris 2005). Alternative plant hosts facilitate the persistence of virus-induced plant disease in the absence of crop hosts by serving as viral reservoirs. Insect vectors of plant viruses use these viral reservoirs as inoculum source for spread to the crop host in subsequent production seasons.

In non-cultivated areas a greater genetic and species diversity of plants, which have often co-evolved with viruses, can be found (Wisler and Norris 2005). As a result of the evolutionary more ancient host-virus interactions, natural hosts are often asymptomatic. With the subsequent introduction of monoculture, immunologically-naïve crops, the virus infects the new host, resulting in a symptomatic infection (Wisler and Norris 2005).

Applied plant virology relies on the detection, monitoring and management of plant viruses in order to limit plant disease (Wilson 2014). As a result of the economic impact of plant viruses on crop hosts, surveillance and research focuses mainly on crop species displaying obvious symptoms, but as discussed above, natural hosts probably represent the major source of new plant viruses and pathogen-host interactions. A recent landscape-scale geo-metagenomic study was conducted across cultivated and non-cultivated areas in two biodiversity hotspots in the Western Cape, South Africa and Rhone River valley, France in order to assess their viromes. Virus incidence was higher in cultivated plants, but 90 new virus species were identified, predominately from non-crop plants (Bernardo et al. 2017). This highlights the importance of also including natural vegetation in surveys aimed at identifying emerging viral diseases of economic importance.

The natural host range for the majority of plant rhabdoviruses described to date is limited to individual species or plant families. MFSV, MMV, CCMoV, WASMV, BYSMV and NCMV all infect plant species in the family *Graminae* (Jackson et al. 2005). The host range of Broccoli

necrotic yellows virus (BNYV) (*Brassicaceae*), FLSV (*Festuca gigantea*), Sonchus virus (SonV) (*Sonchus oleraceus*), SCV (*Fragaria* spp.), RYSV (rice), TaVCV (*Colocasia esculenta*) is also limited to single families or species (Jackson et al. 2005). Viral surveys in wild plant species will enable virologists to determine whether this apparent restricted host range is a characteristic of some species or the result of limited surveillance.

A few members of the cyto- and nucleorhabdoviruses have been reported from multiple hosts (Jackson et al. 2005). These include DYVV (*Datura stramonium*; *Thunbergia alata*), PYDV (*Nicotiana* spp.; *Trifolium* spp.) and LNYV (*Chenopodiaceae*; *Compositae*; *Leguminosae*; *Liliaceae*; *Solanaceae*) (Jackson et al. 2005). EMDV, which has been subject to extensive studies has been reported on *Hibiscus rosa-sinensis*, cucumber, pepper, potato, aubergine, tomato, tobacco, snakemelon, nightshade, *Pittosporum tobira* and *Capparis spinosa* (De Stradis et al. 2008; Kostova et al. 2001; Babaie and Izadpanah 2002; Katis et al. 2011).

### **1.7 Seed, pollen and mechanical transmission**

Viruses reported to be transmitted propagatively such as the reoviruses, marafiviruses, tenuiviruses and rhabdoviruses are generally not transmitted through seed, pollen or mechanical inoculation as viruses transmitted in a propagative manner can persist in the absence of plant hosts (Nault 1997; Hogenhout et al. 2003; Jackson et al. 2005). Seed or pollen transmission has not been described in the *Rhabdoviridae*, but vegetative propagation plays an important role in the spread and maintenance of SCV (infected stolons), PYDV (infected tubers) and EMDV (infected tubers) (Jackson et al. 2005; Pappi et al. 2016). Some of the cereal-infecting plant rhabdoviruses have been shown to be transmitted by vascular puncture (Nault 1997; Hogenhout et al. 2003; Jackson et al. 2005).

Some plant rhabdoviruses such as SbBMV and SYNIV, as well as a suite of unclassified plant rhabdoviruses such as Ivy vein clearing virus (IVCV), Butterbur virus, Soursop yellow blotch virus (SYBV), Pisum virus, Croton vein yellowing virus (CVYV) and Cynara virus are

reported to be transmitted by mechanical abrasion of leaves (Lamprecht et al. 2010; Jackson et al. 2005). Viruses that can be transmitted mechanically often have the capacity to infect a broader range of plants, as they are not restricted to transmission by the vector and thus the host range of the vector. Although it has been reported for some plant rhabdoviruses, direct plant to plant transmission is not considered an important factor in the spread of plant rhabdoviruses (Jackson et al. 2005).

## **1.8 Conclusion**

Plant rhabdoviruses infect a wide range of crop hosts, and the development of disease leads to significant yield losses which limits productivity. The genera *Nucleorhabdovirus* and *Cytorhabdovirus* are of particular interest as members are reported to be transmitted in a persistent, propagative manner by their insect vectors. This allows for their persistence in the absence of other mechanisms facilitating maintenance of viral load and spread such as the presence of alternative plants hosts which function as viral reservoirs, or transmission through seed, pollen or mechanical means. Although some members of the two genera have been subject to extensive studies in order to characterize their interactions with their insect vectors and plant hosts, identify alternative plant hosts and determine their whole genome sequences, others remain largely uncharacterized. A clear understanding of these aspects is required for each plant rhabdovirus-plant host interaction in order to identify and implement control and management strategies to limit production losses associated with these viruses.

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## Tables

**Table 1.1. Plant nucleo- and cytorhabdovirus whole genome sequences available on the National Centre for Biotechnology Information database.**

| <b>Cytorhabdoviruses</b>                    | <b>Genome size</b> | <b>Accession number</b> | <b>Reference</b>                                 |
|---------------------------------------------|--------------------|-------------------------|--------------------------------------------------|
| Lettuce necrotic yellows virus*             | 12 807             | NC_007642<br>AJ867584   | Dietzgen et al. (2006)<br>Dietzgen et al. (2006) |
| Northern cereal mosaic virus                | 13 222             | AB030277                | Tanno et al. (2000)                              |
|                                             | 13 221             | GU985153                | Tanno et al. (2000)                              |
|                                             | 13 222             | NC_002251.1             | Tanno et al. (2000)                              |
| Lettuce yellow mottle virus                 | 12 926             | NC_011532               | Heim et al. (2008)                               |
|                                             |                    | EF687738.1              | Heim et al. (2008)                               |
| Colocasia bobone disease-associated virus** | 12 193             | KT381973                | Higgins et al. (2016)                            |
|                                             | 12 193             | NC_034551.1             | Higgins et al. (2016)                            |
| Barley yellow striate mosaic virus          | 12 706             | KM213865                | Yan et al. (2015)                                |
|                                             | 12 706             | NC_028244.1             | Yan et al. (2015)                                |
| Alfalfa dwarf virus                         | 14 491             | KP205452                | Bejerman et al. (2015)                           |
|                                             | 14 491             | NC_028237.1             | Bejerman et al. (2015)                           |
| Persimmon virus A**                         | 13 467             | NC_018381               | Ito et al. (2013)                                |
|                                             |                    | AB735628.2              | Ito et al. (2013)                                |
| Maize-associated rhabdovirus**              | 11 877             | KY965147                | Willie and Stewart (2017)                        |
| Cabbage cytorhabdovirus 1**                 | 12 949             | KY810772                | Pecman et al. (2017)                             |
| Maize yellow striate virus**                | 12 654             | KY884303.1              | Maurino et al.(2018)                             |
|                                             |                    | KY884672.1              | Maurino et al.(2018)                             |
| Rice stripe mosaic virus**                  | 12 774             | KX525586                | Yang et al. (2017)                               |
| Wuhan insect virus 4**                      | 13 490             | NC_031225.1             | Li et al. (2015)                                 |
|                                             |                    | KM817650.1              | Li et al. (2015)                                 |
| Wuhan insect virus 5**                      | 12 734             | NC_031227.1             | Li et al. (2015)                                 |
| Wuhan insect virus 6**                      | 14 191             | NC_031232.1             | Li et al. (2015)                                 |
|                                             |                    | KM817652.1              | Li et al. (2015)                                 |
| Yerba mate rhabdovirus**                    | 12 876             | KY366322                | Bejerman et al. (2017)                           |
| Strawberry crinkle virus                    | 14 545             | MH129615                | Koloniuk et al (2018)                            |
|                                             | 14 559             | MH129616                | Koloniuk et al (2018)                            |
| Wheat yellow striate virus                  | 14 486             | MG604920                | Liu et al (2018)                                 |
| <b>Nucleorhabdoviruses</b>                  | <b>Genome size</b> | <b>Accession number</b> | <b>Reference</b>                                 |
| Maize fine streak virus                     | 13 782             | AY618417                | Tsai et al. (2005)                               |
|                                             | 13 782             | NC_005974.1             | Tsai et al. (2005)                               |
| Maize mosaic virus                          | 12 133             | AY618418                | Reed et al. (2005)                               |
|                                             | 12 133             | NC_005975.1             | Reed et al. (2005)                               |
| Rice yellow stunt virus                     | 14 042             | AB011257                | Huang et al. (2003)                              |
|                                             | 14 042             | NC_003746.1             | Huang et al. (2003)                              |
|                                             | 14 042             | AB011257.1              | Huang et al. (2003)                              |
| Taro vein chlorosis virus**                 | 12 020             | NC_006942               | Revill et al. (2005)                             |

|                                         |        |                         |                                                  |
|-----------------------------------------|--------|-------------------------|--------------------------------------------------|
|                                         |        | AY674964                | Revill et al. (2005)                             |
| Eggplant mottled dwarf virus            |        |                         |                                                  |
| Agapanthus isolate                      | 13 154 | NC_025389.1             | Babaie et al. (2015)                             |
| Iranian isolate                         | 13 100 | KJ082087                | Zhai and Pappu (2014)                            |
| Iran/SH-eg                              | 13 154 | KC905081.1              | Babaie et al.<br>(2014)(Unpublished)             |
| Datura yellow vein virus                | 13 188 | KM823531<br>NC_028231.1 | Dietzgen et al. (2015)<br>Dietzgen et al. (2015) |
| Potato yellow dwarf virus*              | 12 875 | GU734660                | Ghosh et al. (2008)                              |
| strain CYDV-constricta                  | 12 792 | KY549567.1              | Jang and Goodin<br>(2017) Unpublished)           |
|                                         | 12 875 | NC_016136.1             | Ghosh et al. (2008)                              |
| Iranian maize mosaic virus              | 12 381 | DQ186554                | Massah et al. (2008)                             |
|                                         | 12 426 | NC_036390.1             | Ghorbani et al. (2018)                           |
|                                         | 12 426 | MF102281.1              | Ghorbani et al. (2018)                           |
| Sonchus yellow net virus                | 13 720 | L32603                  | Zuidema et al. (1986)                            |
| (Sowthistle yellow vein virus)          | 13 720 | NC_001615.3             | Scholthof et al. (1994)                          |
| Black currant<br>nucleorhabdovirus 1**  | 14 432 | MF543022.1              | Wu et al. (2018)                                 |
| Physostegia chlorotic mottle<br>virus** | 13 321 | KY706238.1              | Knierim et al. (2018)                            |
|                                         | 13 320 | KX636164.1              | (Unpublished)                                    |
|                                         | 13 321 | KY859866.1              | Menzel et al. (2018)                             |
|                                         |        |                         | Gaafar et al. (2018)                             |

\*Type species; \*\* No ICTV genus-level classification

## Figures

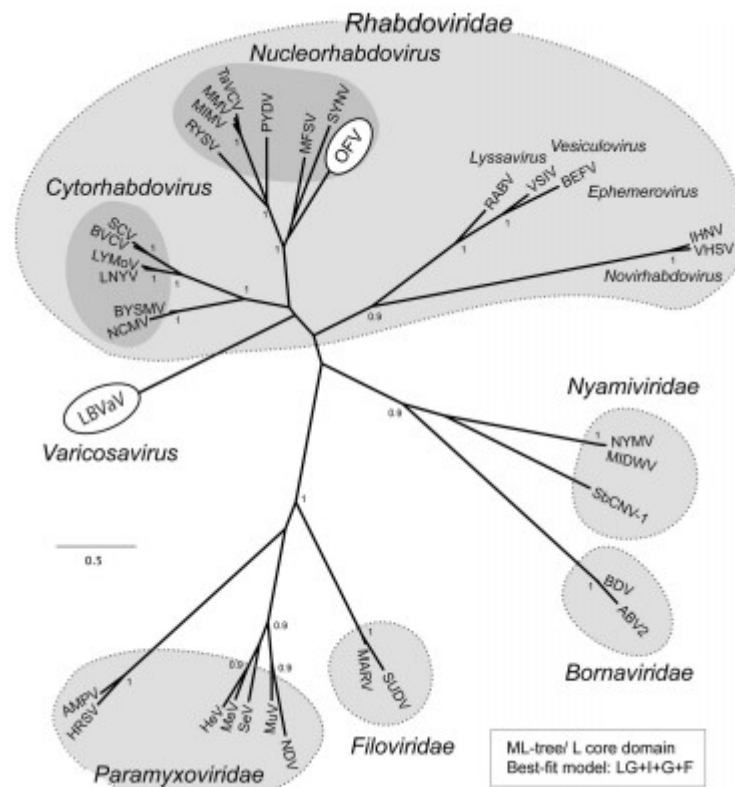


**Figure 1.1.** Evolutionary relationships of some members of the order *Mononegavirales* based on RNA-dependent RNA polymerase sequences corresponding to the core domain. Phylogenetic relationships of two new rhabdovirus genera with bipartite genomes and lacking lipoprotein membranes [*Dichorhavirus* (Orchid fleck virus (OFV)) and *Varicosavirus*] with other genera in the family *Rhabdoviridae* are also shown. Image obtained from Dietzgen et al. (2014).

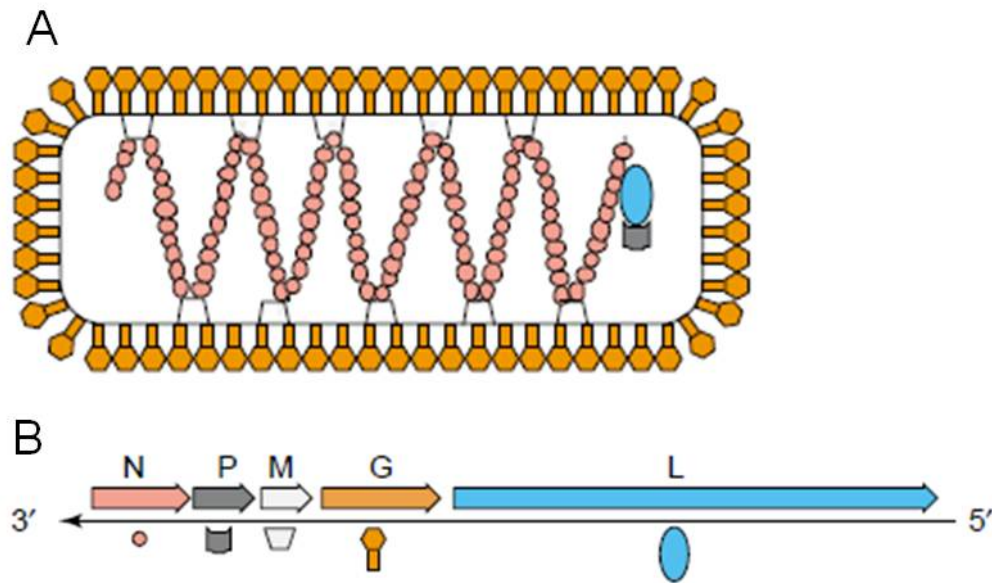


**Figure 1.2.** Minimal virion (**A**) and genome structure (**B**) of rhabdoviruses. A: The viral genomic RNA is coated by nucleocapsid (N) proteins, and is attached to the envelope through the matrix (M) proteins. The polymerase (L) protein is associated with the phosphoprotein (P) and nucleocapsid. G-protein spikes protrude from the envelope. B: Rhabdovirus genomes encode five structural proteins, the N, P, M G and L proteins. Plant rhabdovirus genomes also encode additional ORFs at position X between P and M. Image obtained from Hogenhout et al. (2003).

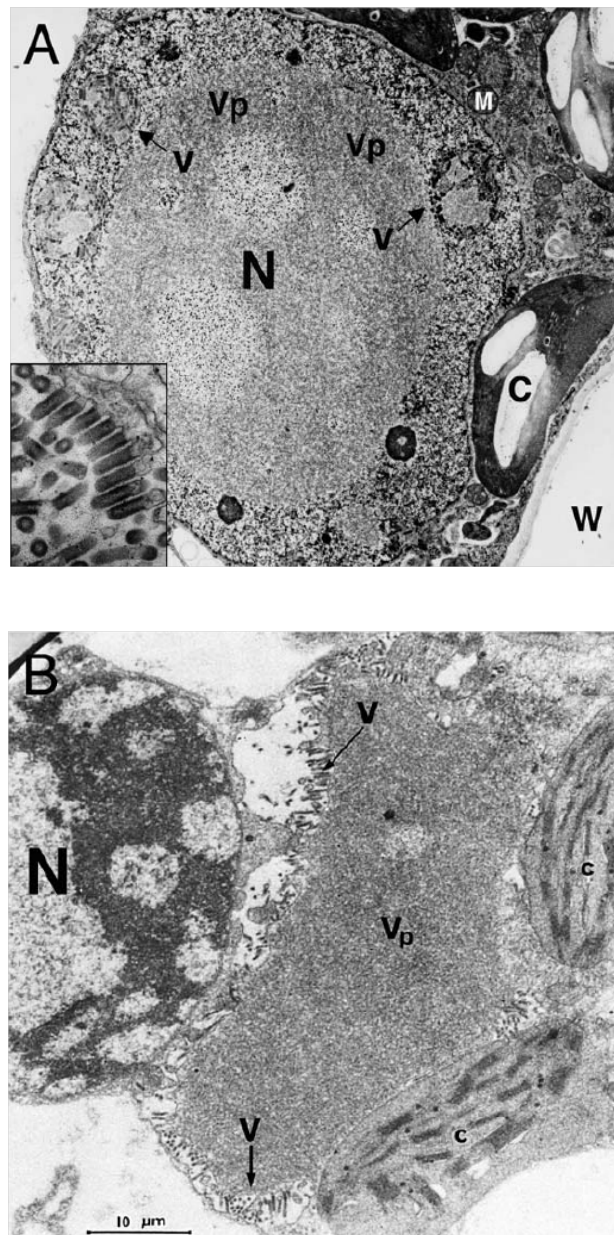


**Figure 1.3.** Electronmicrographs of sites of cyto- and nucleorhabdovirus budding and accumulation in host cells. **A:** Nucleorhabdovirus viroplasms form in enlarged nuclei, and budding occurs at the periphery of the nucleus from the inner nuclear envelope to accumulate in the perinuclear space. **B:** Cytorhabdovirus viroplasms form in the cytoplasm, and budding occurs. V: viral budding; Vp: viroplasms; N: nucleus; C: chloroplast; M: mitochondria; W: cell wall. Image obtained from Jackson et al. (2005).

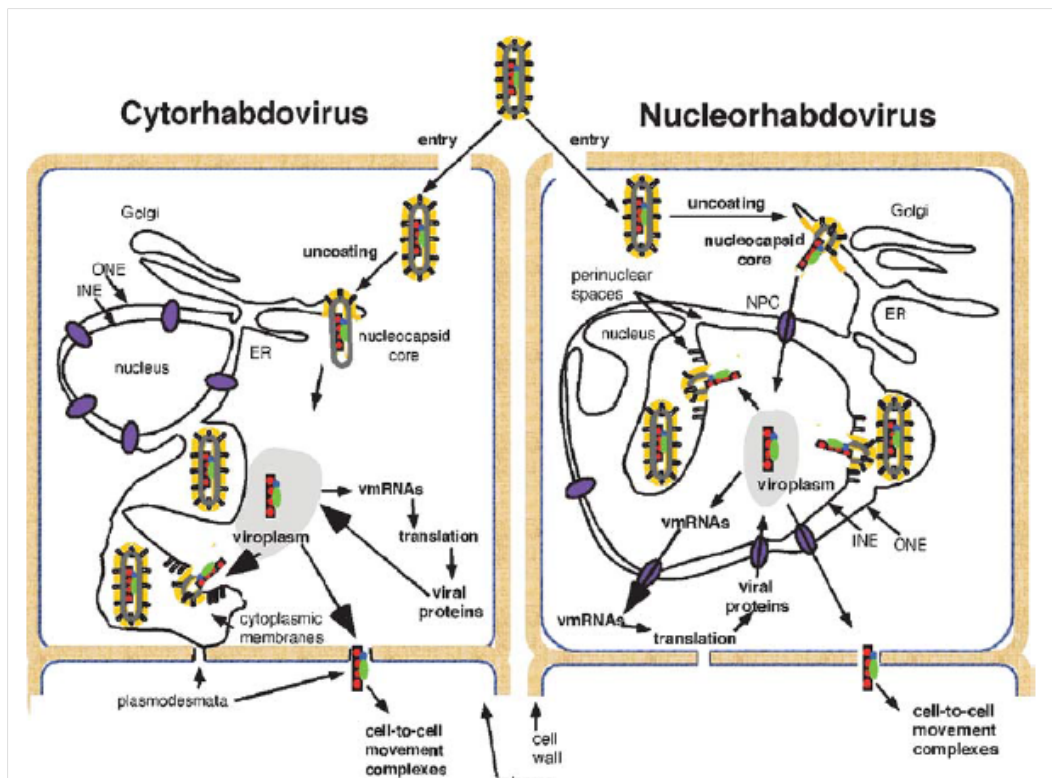


**Figure 1.4.** Replication and virion maturation of cyto- and nucleorhabdoviruses. Following entry into hosts cells by insect feeding and receptor-mediated endocytosis, uncoating occurs on endoplasmic membranes (ER), and nucleocapsids are released into the cytoplasm. From this point onwards, replication, transcription and maturation of virions of cyto- and nucleorhabdoviruses differ. Cytorhabdovirus cores become transcriptionally active and associate with the ER, leading to the formation of viroplasms, where viral mRNAs (vmRNAs) are transcribed and genomic and antigenomic vmRNAs are produced by replication. Translation of vmRNAs occurs at free polyribosomes, and viral proteins involved in replication accumulate in the viroplasms, while viral glycoproteins are targeted to membranes (cytoplasmic or outer nuclear envelope (ONE)). Maturation of viral particles occurs through matrix (M) protein condensation of cores at sites of glycoprotein (G) protein accumulation. For the nucleorhabdoviruses, nucleocapsid cores are imported into the nucleus through nuclear pore complexes (NPC) and transcription occurs. Viral mRNAs are exported into the cytoplasm and translated, and viral proteins are imported into the nucleus. Viral proteins contribute to replication and the formation of viroplasms in the nucleus. Morphogenesis occurs through budding through the INE, leading to expansion of the outer

nuclear envelope). Alternatively, the inner nuclear envelope invaginates to form intranuclear spherules, into which budding occurs for virions to obtain a host derived lipid membrane. Image obtained from Jackson et al. (2005).

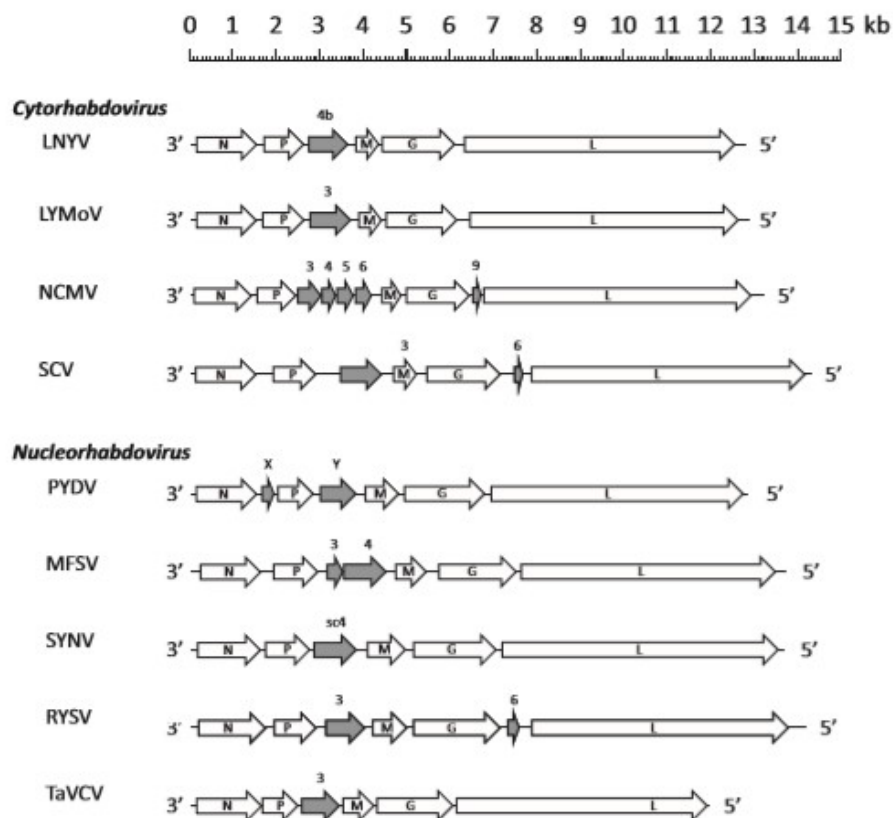


**Figure 1.5.** Genome organization (3'-5') of negative sense plantrhabdovirus genomes. N: nucleocapsid protein gene; P: phosphoprotein gene; X: putative movement protein gene and unknown genes of plant rhabdoviruses; M: matrix protein gene, G: glycoprotein gene, Y: unknown genes of plant and animal rhabdoviruses; L polymerase protein gene. Image obtained from Walker et al. (2011).

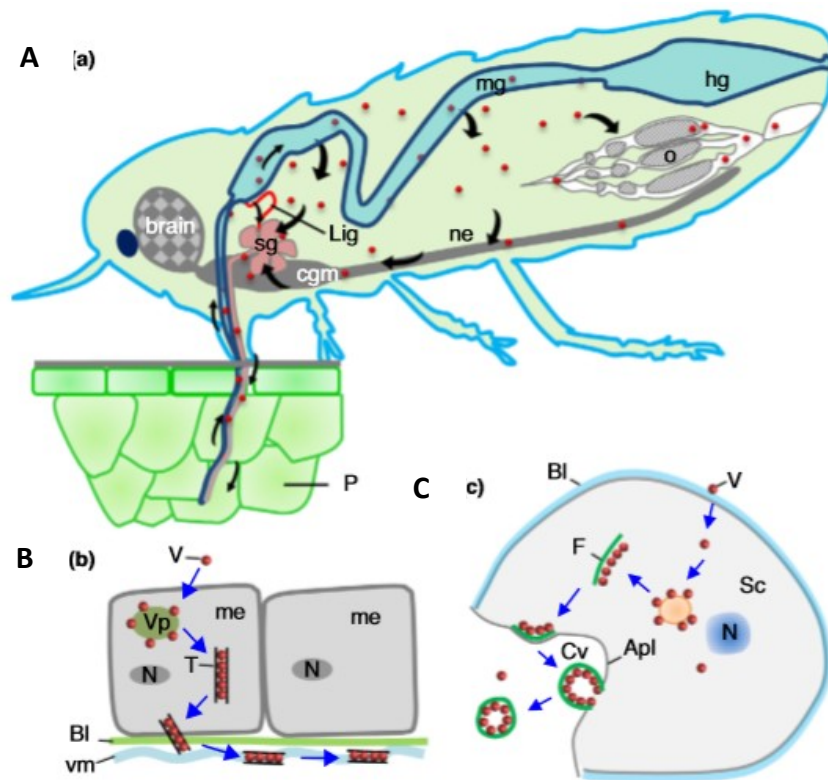


**Figure 1.6.** Proposed mechanisms of viral spread in insect vectors. **A:** After ingestion and entry through receptor-mediated endocytosis, virions cross the basal lamina to move from the midgut epithelium to the salivary glands via the hemolymph, nervous system or ligament-like structures. **B:** Virus-containing tubules are used by plant reoviruses to facilitate movement of virions across the basal lamina. **C:** Entry into salivary glands mediated by exocytosis triggered by virus-induced filaments. Apl: apical plasmalemma; Bl: basal lamina; cgm: compound ganglionic mass; Cv: cavity; F: filaments; hg: hindgut; Lig: ligament-like structures; me: midgut epithelium; mg: midgut; N: nucleus; ne: nerves; O: ovary; P: phloem; Sc: salivary cytoplasm; sg: salivary gland; T: tubule; V: virus particle; vm: visceral muscle; Vp: viroplasm. Image obtained from Jia et al. (2018).

## **Chapter 2**

# **Alternative hosts and seed transmissibility of Soybean blotchy mosaic virus**

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## 2.1 Abstract

Soybean blotchy mosaic virus (SbBMV) is an important virus of soybean in the warmer regions of South Africa. The presence of the virus is associated with blotchy mosaic symptoms on soybean leaves and significant annual yield losses. The virus is a member of the genus *Cytorhabdovirus* and persists between soybean growing seasons. In this study, multiple specimens of indigenous tree species, other crops and herbaceous weeds surrounding soybean fields with high disease incidences of SbBMV were tested for the presence of SbBMV by RT-PCR in order to determine whether the presence of alternative hosts facilitates the seasonal carry-over of the virus. Commercial soybean cultivars commonly grown in the region were also evaluated for seed transmissibility of the virus. A total of 487 accessions representing 27 different species were screened and one accession each of *Flaveria bidentis*, *Lamium amplexicaule* and *Gymnosporia buxifolia* tested positive for the presence of SbBMV and may serve as possible alternative hosts of SbBMV, allowing over-wintering of the virus when soybean is absent. Symptoms associated with SbBMV infection were not present in any of the 2, 829 seedlings collected from naturally infected SbBMV plants, and none of the 21 seedlings showing various abnormalities and tested by RT-PCR were positive. SbBMV does not appear to be seed transmissible in soybean at an incidence above that which numbers screened would have detected. It was concluded that the presence of alternative plant hosts, functioning as viral reservoirs during the soybean off-season might allow for the re-emergence of the disease early in the soybean production season each year. Future work will investigate the role of *Peragallia caboverdensis*, the leafhopper vector of SbBMV, and specifically the possible propagative transmission of the virus in the persistence of the disease.

**Keywords:** plant rhabdovirus, alternative hosts; seed transmissibility; soybean.

Soybean blotchy mosaic virus (SbBMV) is an economically significant viral pathogen of soybean in South Africa first identified in the lower lying soybean production areas in the

northern and eastern parts of South Africa (Pietersen 1993; Pietersen et al. 1998). A high incidence of diseased soybean plants displaying the characteristic blotchy mosaic symptoms on leaves is commonly present early in the production season, with symptom severity declining with time. Surveys conducted in the early 1990s reported disease incidences ranging between 0% and 32%, leading to yield losses of up to 20% depending on the cultivar (Pietersen 1993). In a second study conducted later in the 1990s, disease incidence had risen to between 10% and 50% in fields (Pietersen et al. 1998).

Typical of the cytorhabdoviruses, SbBMV has large, enveloped bacilliform particles, which are distributed in the cytoplasm of host cells (Lamprecht et al. 2010). RT-PCR amplification and sequencing of a 522 nt portion of the RNA-dependent RNA polymerase (*L*) gene confirmed that the new soybean virus was related to the cytorhabdoviruses, with the highest nucleotide similarity (60.7%) to Northern cereal mosaic virus. The virus was successfully transmitted mechanically to both soybean and *Nicotiana benthamiana*, and the leafhopper *Peragallia caboverdensis* Lindberg (*Cicadellidae*, *Agalliinae*) identified as its insect vector (Lamprecht et al. 2010).

Rhabdovirus genomes are generally between 12.5 kb and 14 kb in size, with the monopartite, negative sense genomic RNA encoding five conserved structural proteins, the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and L protein (Dietzgen and Kuzmin 2012; Jackson et al. 2005). Rhabdoviruses in the genera *Nucleorhabdovirus* and *Cytorhabdovirus* also encode one or more accessory genes, postulated to be involved in movement between cells and other unknown functions. Recently, the family *Rhabdoviridae* was expanded to include two additional plant-infecting genera, the *Dichorhavirus* and *Varicosavirus*, which have bipartite genomes (Amarasinghe et al. 2017).

Despite the annual nature of soybean production, this disease of soybean persists between soybean growing seasons, as symptoms are present early in each new growing season.

Possible mechanisms allowing for the carry-over of the virus between seasons include the presence of alternative plant hosts in areas surrounding soybean fields, presence of soybean volunteer plants during winter and/or replicative transmission of the virus by its insect vector. Seed or pollen transmission is another method by which the virus can be introduced each year, especially as high incidences of the disease are often observed early in the growing season.

Virus transmission through seed has been observed in at least 25 groups of plant viruses, with approximately 18% of all plant viruses reportedly transmitted through seed (Johansen et al. 1994; Maule and Wang 1996; Mink 1993). Although only a small percentage of infections result in eventual seed transmission, virus propagation through this mechanism is significant as a result of the introduction of new viral sources for further spread through arthropod vectors. Seed transmission, however, plays a significant role in the epidemiology of only a few plant viruses such as Bean common mosaic virus in beans (Morales and Castano 1987), Soybean mosaic virus in soybean (Bowers and Goodman 1979), Lettuce mosaic virus in lettuce (Dinant and Lot 1992), Cucumber mosaic virus (CMV) (Johansen et al. 1994; Mink 1993), and the maize lethal necrosis complex (Mahuku et al. 2015; Zhang et al. 2011), but to date seed or pollen transmission of rhabdoviruses has not been reported (Jackson et al. 2005).

In this study, the presence of alternative plant hosts and seed transmissibility of SbBMV was investigated for the first time. Asymptomatic indigenous trees, other crop plants and herbaceous weeds commonly present in areas surrounding fields where high disease incidences are observed each year were sampled and tested for the presence of SbBMV by RT-PCR. Leaf samples were collected from trees, crops, grasses and other herbaceous weeds in close proximity to commercial soybean fields with a disease incidence of at least 30% in the Brits area of the North West province in South Africa. Leaf material was collected at multiple sites on a plant, and each sample was assigned a unique accession number and GPS coordinates were documented for trees. Voucher specimens were collected for all plant

species surveyed, and were identified by expert botanists in the Department of Plant Sciences, University of Pretoria, South Africa. Samples were collected in both the soybean production and off-season, and leaf material was stored in plastic bags at 4°C prior to being processed.

Pooled leaf samples (pieces from multiple individual leaves) were homogenized in liquid nitrogen, and total RNA extracted according to the method described by White et al. (2008) with a few modifications which included the omission of spermidine from the extraction buffer, and the use of 1.8 ml heated extraction buffer per sample. All centrifugation steps on the first day were performed for 15 min, and RNA pellets were washed with 500 µl 70% ethanol. The plant gene ribulose 1,5-biphosphate carboxylase (*RuBisCo*) was amplified from RNA of all accessions using the *rbcLa* primer pair to ensure RNA quality is sufficient for RT-PCR analysis as polysaccharides, polyphenolics and other secondary metabolites associated with woody tissues can bind or co-precipitate RNA, lowering RNA quality and leading to false negative results (White et al. 2008). cDNA synthesis was done using the M-MLV Reverse Transcriptase system (Promega, Madison, USA) according to the manufacturer's instructions with two modifications to increase primer concentration (primer volume increased and no water added to primer/RNA template): 5 µL random hexamer primer (4 µM primer in final reaction) was allowed to anneal to 2 µl total RNA. This was followed by the addition of 5.5 µl master mix consisting of 2.5 µl M-MLV RT 5x Reaction buffer (Promega), 1.25 µl dNTPs (10 mM each dATP, dCTP, dGTP, dTTP) (Kapa Biosystems, Wilmington, USA), 0.125 µl (25 U) M-MLV RT (Promega) and 1.6 µl molecular grade water added for cDNA synthesis. Each 12.5 µl PCR amplification reaction contained 2.5 µl 5x MyTaq Reaction buffer (Bioline, London, UK), 0.25 µl (0.2 µM) each of *rbcLa* F (5' ATGTCACCACAAACAGAGACTAAAGC 3') (Levin et al. 2007) and *rbcLa* R (5' GTAAAATCAAGTCCACCRCC 3') (Kress and Erickson 2007), 0.125 µl (0.6 U) MyTaq DNA Polymerase (Bioline), 1.5 µl cDNA and molecular grade water to 12.5 µl. The PCR program consisted of 95°C for 1 min, followed by 35 cycles of 95°C for 15 sec, 55°C for 15 sec, 72°C

for 10 sec, and a final extension step at 72°C for 10 min. PCR products were separated by agarose gel electrophoresis on ethidium bromide-stained gels (1%), and visualized using UV light.

SbBMV was detected in leaf samples by RT-PCR using the Soyblotch F (5' CTTTGCCCAACTGGACTCCC 3') and Soyblotch R (5' TCCAAACAGTCTTCCCAGGC 3') primer pair designed to amplify a 354 bp portion of the SbBMV *L* gene, for which sequence information was available on the National Centre of Biotechnology Information (NCBI) database (EU877231). RT-PCR reactions were done in duplicate. cDNA synthesis was performed using the M-MLV Reverse Transcriptase system (Promega) as described above, with the Soyblotch F primer at 4 µM final concentration. PCR amplification was done using the MyTaq system (Bioline) as described above using the Soyblotch F and Soyblotch R primer pair, and an annealing temperature of 58°C. Soyblotch PCR products were purified for direct sequencing through the addition of 5 U Exonuclease I (Thermo Scientific, USA) and 1 U FastAP Alkaline Phosphatase (Thermo Fisher, Waltham, USA) to 7 µl PCR product, and incubated at 37°C and 85°C for 15 min each. Each 10 µl sequencing reaction consisted of 3 µl template, 1 µl BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Foster City, USA), 2.25 µl of 5x Sequencing buffer (Applied Biosystems) and 2 µM (0.75 µl) of the Soyblotch F and Soyblotch R primers respectively. Sequences were subjected to a Basic Local Alignment Search Tool (BLASTn) search in NCBI in order to confirm presence of SbBMV.

A total of 487 specimens representing 27 different species were collected in the soybean growing season in and surrounding fields where the disease incidence exceeded 30% (Table 2.1). Of these, two accessions, one each of *Flaveria bidentis* (Smelter's bush) and *Gymnosporia buxifolia* (spike thorn) consistently tested positive for the presence of the virus. Some samples were also collected during the South African winter months, from the same sites where samples had been collected during the soybean growing season. In this period, maize or wheat is commonly grown in rotation with soybean in the Brits area in South Africa.

The accession of *Lamium amplexicaule* (henbit) which tested positive for the presence of SbBMV was collected during winter, confirming the presence of the virus in an alternate host in the absence of soybean.

Two of the three new putative hosts identified for SbBMV were species of common weed. Weeds can act as pests, vector pathogens or act as reservoir hosts for viruses and insects as in the case of henbit and Smelter's bush and SbBMV (Wisler and Norris 2005). The higher genetic diversity of weeds in comparison to crops, the presence of numerous different weed species in an area, which limits contact between identical, susceptible plants, and long term selection of tolerance or resistance often results in asymptomatic virus infection in weed hosts. As a result of the establishment of a new monoculture crop, pathogens which were present asymptotically at low levels in weeds may jump host to the new introduced crop, resulting in a symptomatic infection (Wisler and Norris 2005). It is possible that SbBMV is re-introduced onto soybean annually in this manner.

Henbit is a winter annual which germinates in late summer and flowers and dies the following spring or summer, and may thus act as a "bridge" for SbBMV between successive soybean seasons. Henbit has also previously been reported to serve as a naturally infected weed host for plant viruses such as Turnip yellows virus (Stevens et al. 2008), CMV (Nitzany 1975), and Turnip mosaic virus (Stobbs and Stirling 1990). Potato virus Y and CMV have also been detected in other species in the genus such as *Lamium purpureum*, indicating that members of the genus may commonly be hosts to plant viruses (Kaliciak and Syller 2009; Tomlinson et al. 1970). Smelter's bush is a member of the *Asteraceae* family, and considered an annual weed, but may survive the winter months in warmer areas such as Brits, where samples were collected. In contrast to henbit, Smelter's bush does not appear to be a common alternative host for plant viruses, but reports of CMV on Smelter's bush are present in literature (Swanepoel and Nel 1995).

Spike thorn is an indigenous tree species commonly found in those areas where high incidences of the blotchy mosaic symptom is found, such as the Brits, Loskop Irrigation Scheme and Groblersdal areas in South Africa. As with henbit, spike thorn is a member of the family *Fabaceae* (*Leguminosae*), and it was considered likely that SbBMV could move from one leguminous host to the next. The perennial nature of spike thorn, in contrast to the annual or short-lived perennial weeds henbit and Smelter's bush might render it a more effective and permanent reservoir host.

Seed from naturally infected, RT-PCR positive commercially grown soybean plants were also collected and planted to assess the seed transmissibility of the virus. This disease of soybean emerges early in the summer soybean growing season each year following the soybean off-season in the winter months. The early appearance of symptoms in a season is commonly observed when viruses are seed transmissible. 11, 6, 10 and 14 soybean plants of the soybean cultivars PHB 94Y80R (Pioneer), PHB 94Y20R (Pioneer), NS 7211R (Klein Karoo Seed Marketing) and 6.15F (Southern Hemisphere Seeds) respectively displaying the characteristic blotchy mosaic associated with SbBMV infection in fields were marked, and leaf material collected. The presence of SbBMV in leaf material was confirmed by RNA extraction and RT-PCR using the Soyblotch primer pair as described above. Seed was harvested once the pods had dried off, inoculated with *Rhizobium* spp. and grown in steam-sterilized potting mix under insect-free greenhouse conditions. Seedlings were watered approximately three times per week, and were visually evaluated for symptoms when they had reached at least the trifoliate stage. Seedlings displaying any virus-like symptoms or abnormalities were tested for SbBMV by RT-PCR using the Soyblotch RT-PCR system.

A total of 2, 829 seedlings representing four commercial cultivars commonly grown in the warmer, low-lying soybean production areas in South Africa were evaluated for symptoms. For each of the soybean cultivars 6.15F, PHB 94Y20R, PHB 94Y80R and NS7211R, 983, 125, 965, and 756 seedlings were screened respectively. The characteristic blotchy mosaic typically associated with the presence of SbBMV (Figure 2.1A) was not observed in any of

the seedlings (Figure 2.1B). None of the 21 seedlings displaying various abnormalities and tested by RT-PCR were positive for SbBMV. SbBMV does not appear to be seed transmissible in these soybean cultivars at levels high enough to be detected given the numbers tested. This was expected, as no rhabdoviruses have been shown to be seed transmissible to date (Jackson et al. 2005; Dietzgen et al. 2017).

In future, control strategies against SbBMV should focus on the management of sources of inoculum from which the virus can be spread to soybean by *P. caboverdensis*. Weeds and volunteer plants can be eliminated, and crop residues destroyed (Wisler and Norris 2005). Removal of Johnson's grass, an alternative host of Maize dwarf mosaic virus through the application of herbicides increased yields of corn (Mark and Harold 1993). Treatment of perennial hosts such as trees with systemic insecticides will prevent *P. caboverdensis* and any other possible insect vectors from re-infesting soybean. The apparent lack of seed transmissibility of SbBMV implicates *P. caboverdensis* as the main route by which the virus spreads. Future work should include further characterization of the role of *P. caboverdensis* in the epidemiology of SbBMV, such as associations with alternative hosts and mode of transmission of SbBMV, as well as the presence of additional insect vectors.

## **2.2 Acknowledgements**

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## Tables

**Table 2.1. List of plant species collected and results of PCR tests for Soybean blotchy mosaic virus.**

| <b>Botanical name</b>             | <b>Common name</b>         | <b>Nr positive/nr tested</b> |
|-----------------------------------|----------------------------|------------------------------|
| <i>Amaranthus hybridus</i> (A)    | Smooth pigweed             | 0/29                         |
| <i>Bidens pilosa</i> (A)          | Blackjack                  | 0/16                         |
| <i>Celtis africana</i>            |                            | 0/17                         |
| <i>Chenopodium album</i>          | Goosefoot/lambsquarters    | 0/16                         |
| <i>Chenopodium corinatum</i> (A)  | Creeping goosefoot         | 0/33                         |
| <i>Cyperus</i> spp.               | -                          | 0/23                         |
| <i>Datura stramonium</i>          | Jimson weed; Thorn apple   | 0/31                         |
| <i>Dichrostachys cinerea</i> (P)  | Sickle bush                | 0/15                         |
| <i>Dinebra retroflexa</i> (A)     | Viper grass                | 0/25                         |
| <i>Euphorbia heterophylla</i>     | Poinsettia                 | 0/20                         |
| <i>Flaveria bidentis</i> (A)      | Smelter's bush; Yellowtops | 1/27                         |
| <i>Gymnosporia buxifolia</i> (P)  | Spike thorn                | 1/26                         |
| <i>Ipomoea purpurea</i>           | Morning glory              | 0/5                          |
| <i>Lamium amplexicaule</i>        | Henbit                     | 1/10                         |
| <i>Malvastrum coromondelianum</i> | False mallow               | 0/13                         |
| <i>Medicago sativa</i>            | Lucerne                    | 0/29                         |
| <i>Portulaca quadrifida</i> (A)   | Chickenweed                | 0/32                         |
| <i>Rapistrum rugosum</i>          | Bastard cabbage            | 0/24                         |
| <i>Searsia leptodictya</i> (P)    | Mountain karee             | 0/10                         |
| <i>Senecio abiifolius</i>         | -                          | 0/3                          |
| <i>Senegalia caffra</i> (P)       | Hook thorn                 | 0/10                         |
| <i>Sonchus oleraceus</i> (A)      | Sowthistle                 | 0/9                          |
| <i>Sonchus</i> spp                | Sowthistle                 | 0/2                          |
| <i>Symbrium thellungi</i>         | -                          | 0/3                          |
| <i>Tithonia rotundifolia</i> (A)  | Red sunflower              | 0/12                         |
| <i>Vachellia karroo</i> (P)       | Sweet thorn                | 0/37                         |
| <i>Vachellia tortilis</i> (P)     | Umbrella thorn             | 0/10                         |

<sup>P</sup>Perennial, <sup>A</sup>Annual

## Figures

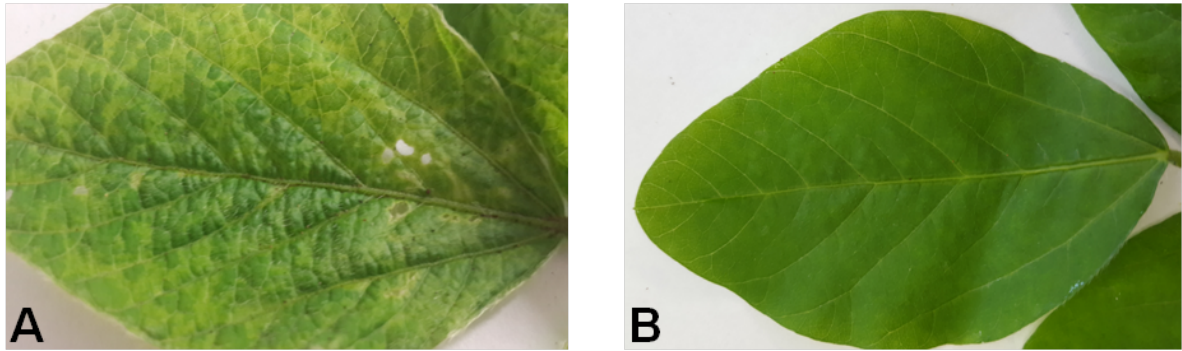


**Figure 2.1.** Photographs illustrating **A)** the typical blotchy mosaic associated with Soybean blotchy mosaic virus on soybean leaves, and **B)** absence of symptoms on accessions grown from seed collected from diseased soybean mother plants in the field.

## **Chapter 3**

# **Development of a strand-specific RT-PCR to detect the positive sense replicative strand of Soybean blotchy mosaic virus**

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### 3.1 Abstract

Soybean blotchy mosaic virus (SbBMV), a plant virus of the genus *Cytorhabdovirus* is an economically important virus of soybean reported only from the warmer, lower-lying soybean production areas in South Africa. The virus consistently appears in soybean crops annually in spite of the absence of soybean plants in winter. One possible reason for this may be that the virus replicates and hence persists in the SbBMV vector, a leafhopper, *Peragallia caboverdensis*. RNA viruses with antisense genomes as inferred for SbBMV produce positive sense RNAs as intermediate replicative forms during replication in their hosts, and detection of the positive strand in the plant host or vector is evidence of virus replication. In this study, a positive strand-specific-RT-PCR (pss-RT-PCR) was developed to detect the positive RNA strand of SbBMV and validated on nine SbBMV isolates from soybean. The effect of tagged reverse transcription (RT) primers for cDNA synthesis, coupled with PCR using a tag-specific primer, as well as removal of unincorporated RT primers following cDNA synthesis was assessed. The positive RNA strand of SbBMV in infected plants was successfully detected following this protocol. Reverse transcription with forward and unmodified reverse primers confirmed that the assay was not able to detect the genomic sense RNA or self-primed cDNAs, lacking the non-viral tag, respectively. However, Exonuclease I (Exo I) treatment of cDNA was required to eliminate false-positive results during PCR amplification.

**Keywords:** replication; rhabdovirus; positive strand-specific RT-PCR

### 3.2. Introduction

Soybean blotchy mosaic virus (SbBMV) is a single-stranded, negative sense RNA virus of the genus *Cytorhabdovirus* within the family *Rhabdoviridae*. SbBMV was first detected in the North-West, Limpopo and Mpumalanga provinces of South Africa during surveys conducted to identify viruses of soybean in the 1990's (Pietersen and Garnett 1990; Pietersen 1993; Pietersen et al. 1998). Despite the annual nature of soybean production, infected leaves

display the characteristic blotchy mosaic symptoms early in the season each year, with symptoms declining with time. Isolation and characterization led to the identification of bacilliform-shaped virions distributed in the cytoplasm of host cells, leading the authors to hypothesizing that the virus was a member of the cytorhabdoviruses (Lamprecht et al. 2010). This was confirmed by phylogenetic analysis when the nucleotide sequence of a 522 nt portion of the RNA-dependent RNA polymerase (L) gene was also determined. The leafhopper *Peragallia caboverdensis* (Lindberg) was identified as the insect vector of SbBMV (Lamprecht et al. 2010).

Four plant-infecting genera, *Cytorhabdovirus*, *Nucleorhabdovirus*, *Dichorhavirus* and *Varicosavirus* are distinguished within the family *Rhabdoviridae* (Amarasinghe et al. 2017). Dichorha- and varicosaviruses have two genome segments, while cyto- and nucleorhabdoviruses have a single genomic RNA and are classified based on their intracellular location and sites of replication (Jackson et al. 2005). The linear genomes of rhabdoviruses are between 12 and 14.5 kb in size, and encode five structural proteins, the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and L protein (Jackson et al. 2005; Dietzgen and Kuzmin 2012). In addition, plant nucleo- and cytorhabdovirus genomes also contain between one and four additional open reading frames at position X between the P and M genes, or position Y between the G and L genes, postulated to be involved in movement from cell to cell and other uncharacterized functions.

Members of the *Cytorhabdovirus* and *Nucleorhabdovirus* genera are transmitted in a persistent, propagative manner by their insect vectors which include leafhoppers (*Cicadellidae*), planthoppers (*Delphacidae*) and aphids (*Aphididae*) (Hogenhout et al. 2003). Examples include Maize mosaic virus (Whitfield et al. 2015; Barandoc-Alviar et al. 2016), Barley yellow striate virus, Northern cereal mosaic virus and Wheat chlorotic streak virus (Conti 1985). Positive sense mRNAs corresponding to each gene and full length complements of the genomic RNA are produced during the replication of RNA viruses to serve as mRNA and genomic RNAs for packaging into virions respectively (Plaskon et al.

2009). For an antisense RNA virus such as SbBMV, this would implicate the production of positive sense RNAs which function as mRNA and a replicative intermediate for the production of negative sense genomic RNAs. Strand specific-RT-PCR (ss-RT-PCR) and ss-RT-qPCR is used to detect and quantify these replicative forms during studies of RNA virus replication (Craggs et al. 2001; Gu et al. 2007; Gunji et al. 1994; Komurian-Pradel et al. 2004; Lanford et al. 1994; Lin et al. 2002; Plaskon et al. 2009; Purcell et al. 2006).

Previous studies have reported that standard, unmodified virus-specific primers are not able to differentiate between strand-specific cDNAs transcribed from virus-specific primers or those generated through false priming, especially when the complementary strand is present at high levels (Plaskon et al. 2009; Purcell et al. 2006; Komurian-Pradel et al. 2004; Craggs et al. 2001). cDNA synthesis using strand-specific primers with a non-viral tag sequence at the 5' end incorporates the unique tag into the cDNA. Subsequent PCR with a tag-specific primer results in the specific amplification of cDNAs with the tag sequence, which eliminates amplification and thus detection of cDNAs that are the result of false priming. The use of thermostable reverse transcriptases, such as rTth which allow for reverse transcription at temperatures as high as 70°C, increasing specificity, has also been reported (Craggs et al. 2001; Lanford et al. 1994). Despite the use of tagged primers in reverse transcription and tag-specific primers during PCR, factors such as reverse transcriptase activity or unincorporated tagged reverse primer in the PCR reaction can lead to false positive results (Craggs et al. 2001).

The replication of SbBMV within its vector may represent one of the mechanisms by which the virus persists between growing seasons. In the past, traditional and time intensive techniques such as electron microscopy and serial dilution passages were used to investigate whether viruses were transmitted by their insect vectors in a propagative manner (Nault and Rodriguez 1985; Jackson et al. 2005). The development of a ss-RT-PCR directed at the replicative strands of SbBMV thus offers a fast, efficient tool to study the replication of SbBMV in *P. caboverdensis*.

In this study, the effect of a tagged RT primer and tag-specific PCR primer combination, as well as removal of ssDNA (primers) from cDNA prior to PCR amplification on the specificity of a positive strand-specific-RT-PCR (pss-RT-PCR) for SbBMV was assessed. We report the development of the first pss-RT-PCR for SbBMV. The assay relies on the use of a 5'-tagged antisense primer, of which the 3' end is complementary to the SbBMV *L* gene for reverse transcription, and Exonuclease I (Exo I) digestion of cDNA to remove primers before PCR. PCR amplification is performed using a sense primer specific to the SbBMV *L* gene and a tag-specific antisense primer. This protocol was then tested on nine SbBMV isolates from different temporal and geographical origins in South Africa. An internal control gene was first amplified to confirm RNA quality, and the presence of SbBMV in field samples confirmed using a diagnostic Soyblotch RT-PCR. This was followed by detection of replication using the newly established Soyblotch pss-RT-PCR.

### **3.3. Materials and methods**

To determine the specificity of the Soyblotch pss-RT-PCR for the positive strand of SbBMV, different primers were used for reverse transcription to produce cDNAs of specific polarities to serve as positive and negative controls for the ss-RT-PCR. The Soyblotch F primer was used to prime reverse transcription in order to generate cDNAs of negative polarity, while the Soyblotch R and Soyblotch R-Tag primers were used to produce cDNAs corresponding to the positive sense strand, which is the replicative intermediate of SbBMV. The cDNAs transcribed from the Soyblotch F and Soyblotch R primers served as negative controls for the Soyblotch pss-RT-PCR, as the ss-RT-PCR should be specific for the positive sense strand and positive sense cDNAs containing the non-viral tag sequence. Similarly, reverse transcription in the absence of primer was performed to confirm that it is not possible to amplify cDNAs lacking the non-viral tag sequence using the Soyblotch pss-RT-PCR. cDNAs transcribed using the Soyblotch R-Tag primer served as positive control for the Soyblotch pss-RT-PCR as a result of a positive polarity and the presence of the non-viral tag sequence.

### 3.3.1 Primer design

Soyblotch F and Soyblotch R primers (Table 3.1) were designed to amplify a 354 bp portion of the SbBMV *L* gene sequence (EU 877231.1) available on the National Centre for Biotechnology Information (NCBI) database by RT-PCR, and serves as diagnostic Soyblotch RT-PCR for detection of SbBMV in plants and *P. caboverdensis* (Strydom and Pietersen 2018a, 2018b). In the Soyblotch pss-RT-PCR a modified Soyblotch R primer (Table 3.1) with a 5' tag was used for reverse transcription. The non-viral sequence used as tag on the Soyblotch R-Tag primer and the tag-specific primer was obtained from literature (Lin et al. 2002; Komurian-Pradel et al. 2004). The Soyblotch F and tag-specific reverse primer (Table 3.1) were used for PCR amplification of cDNA transcribed using the Soyblotch R-Tag primer in the Soyblotch pss-RT-PCR. PCR products of the Soyblotch pss-RT-PCR were 387 bp in size.

### 3.3.2 RNA extraction

Infected soybean leaf material in which SbBMV replicates has RNA of both polarities present, and two isolates, 16/4265 and 16/4266 (Table 3.2) were used as positive controls to optimize and test a new pss-RT-PCR for SbBMV. Once established, the protocol was tested on nine other soybean field samples collected from different years and geographic origins in South Africa. Symptomatic leaf material was collected in the field, assigned a unique accession number and stored in plastic bags until processed. Dried leaf material was also obtained from the Virus, Antiserum and Seroreagent (PVAS) collection at the Agricultural Research Council-Plant Protection Research Institute (ARC-PPRI) in Pretoria, South Africa. Leaf material was homogenized in liquid nitrogen, and total RNA extracted according to the method of White et al. (2008) with modifications as described by Strydom and Pietersen (2018a, 2018b). Shortly, 1.8 ml cetyltrimethylammonium bromide (CTAB) buffer (2% CTAB; 2% polyvinylpyrrolidone (PVP) K-40; 25 mM ethylenediaminetetraacetic acid (EDTA); 100 mM Tris-Hydrochloride (Tris-HCL) pH 8; 2 M sodium chloride (NaCl) and 3%  $\beta$ -

mercaptoethanol), heated to 65°C was used per sample. Homogenized leaf material was added to buffer and incubated at 65°C for 30 min with vortexing every 5 min. Supernatant was collected by centrifugation, followed by two sequential chloroform:isoamylalcohol (C:I) (24:1) extractions through the addition of an equal volume C:I and centrifugation. All centrifugation steps on the first day were performed at 11 337 xg for 15 min. Lithium chloride (LiCl) was added to a final concentration of 2M, and incubated at 4°C overnight. The following day, RNA was precipitated by centrifugation for 60 min, the supernatant discarded and the pellet washed with 500 µl 70% ethanol. Ethanol was removed, and pellet resuspended in 50 µl molecular grade H<sub>2</sub>O after air drying.

### **3.3.3 Reverse transcription**

Complementary strand synthesis followed a standard protocol with only different primers utilized. The Soyblotch F, Soyblotch R or Soybotch R-Tag primers were used to initiate reverse transcription in order to test the specificity of the pss-RT-PCR assay. Five µl primer (4 µM primer in final reaction) was allowed to anneal to 2 µl RNA by heating to 70°C for 5 min, and cooling for 5 min at 4°C. 2.5 µl M-MLV Reverse transcriptase 5x Reaction buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT in final working solution) (Promega, Madison, USA), 1.25 µL dNTPs (10 mM each dATP, dCTP, dGTP and dTTP) (Kapa Biosystems, Wilmington, USA), 0.125 µl (25 U) M-MLV Reverse Transcriptase (Promega) and 1.7 µl molecular grade H<sub>2</sub>O to a final volume of 12.5 µl was added to the annealed primer and template. The reaction was incubated at 42°C for 60 min. Reactions were performed in duplicate, with one reaction subjected to Exo I digestion following reverse transcription to remove any remaining RT primer to evaluate the effect of unincorporated primers on the specificity of the Soyblotch pss-RT-PCR assay. This involved cDNA (12.5 µl) being incubated with 1 µl Exo I (20 U) (Thermo Scientific, Waltham, USA) at 37°C for 15 min, followed by incubation at 85°C for 15 min.

#### **3.3.3.1 Reverse transcription of the genomic (negative) strand of SbBMV**

The Soyblotch F primer was used in cDNA synthesis for the diagnostic Soyblotch RT-PCR to confirm the presence of SbBMV in soybean plant material. cDNAs corresponding to the genomic RNA of 16/4265 and 16/4266 served as negative control in Soyblotch pss-RT-PCR optimization.

#### **3.3.3.2 Reverse transcription of the antigenomic (positive) strand of SbBMV**

The Soyblotch R and Soyblotch R-Tag primers were used to transcribe the positive (replicative) strand of the SbBMV *L* gene. The unmodified and tagged Soyblotch R primers were compared for use in detecting virus-specific RNAs.

#### **3.3.3.3 Reverse transcription of falsely primed cDNAs**

To assess the effect of non-specific priming during reverse transcription and the specificity of the Soyblotch pss-RT-PCR for cDNAs with the non-viral tag, cDNA was produced as described above with primer replaced with water.

#### **3.3.3.4 Reverse transcription of internal control gene**

As internal control for RNA quality, the plant gene ribulose 1,5-bisphosphate carboxylase (*RuBisCo*) was amplified from RNA of all isolates using the *rbcLa* primer pair (Levin et al. 2007; Kress and Erickson 2007) following cDNA synthesis using random hexamer primers.

#### **3.3.4 PCR amplification and Sanger sequencing**

Each 25 µl PCR amplification reaction consisted of 2 µl cDNA, 5 µl MyTaq Reaction buffer (1 mM dNTPs, 3 mM MgCl<sub>2</sub>) (Bioline, London, UK), 0.5 µl (0.2 µM) forward primer, 0.5 µl (0.2 µM) reverse primer, 0.25 µl (1.25 U) MyTaq DNA polymerase (Bioline) and 16.8 µl molecular grade H<sub>2</sub>O. PCR cycling conditions for the Soyblotch RT-PCR and pss-RT-PCR consisted of an initial denaturation step at 95°C for 1 min, followed by 35 cycles of 95°C for 15 sec, 58°C for 15 sec and 72°C for 10 sec. Final extension of PCR products took place at 72°C for 5

min. PCR products (6 µl) were separated by agarose gel electrophoresis on ethidium bromide-stained 2% agarose gels, and viewed under UV light. PCR products were purified for sequencing through the addition of 0.5 µl (10 U) Exo I (Thermo Scientific) and 2 µl (2 U) FastAP Alkaline Phosphatase (Thermo Scientific) to each 19 µl PCR product. This was followed by two 15 min incubation steps, first at 37°C, followed by incubation at 85°C. Each 10 µl sequencing reaction consisted of 1 µl template DNA, 1 µl of BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Foster City, USA), 2.25 µl 5x Sequencing buffer (Applied Biosystems) and 2 µM of each primer. Sequences obtained were used in Basic Local Alignment Search Tool (BLASTn) searches on the NCBI database.

#### **3.3.4.1 Diagnostic Soyblotch RT-PCR**

The Soyblotch F and Soyblotch R primers were used in the diagnostic Soyblotch RT-PCR to confirm the presence of SbBMV in field samples. To compare the specificity in detection between the diagnostic Soyblotch RT-PCR and the Soyblotch pss-RT-PCR, cDNAs transcribed in the absence of primer was also subjected to the Soyblotch RT-PCR. The PCR product of the diagnostic Soyblotch RT-PCR is 354 bp in size.

#### **3.3.4.2 Soyblotch pss-RT-PCR**

To investigate the possible increased specificity in detection conferred by the Soyblotch R-Tag RT primer, cDNA transcribed from both the standard and modified Soyblotch R primer were subjected to PCR amplification using the Soyblotch F and tag-specific primer. cDNAs transcribed from the Soyblotch F primer and in the absence of RT primer were also subjected to the Soyblotch pss-RT-PCR to confirm the specificity of the Soyblotch pss-RT-PCR for the positive strand and to compare differences in the specificity of detection between the diagnostic Soyblotch RT-PCR and the Soyblotch pss-RT-PCR respectively. PCR amplification of cDNAs transcribed in the absence of primer, together with the corresponding positive controls was performed at 66°C in order to eliminate low-level

amplification in the Soyblotch pss-RT-PCR. The expected amplicon size of the Soyblotch pss-RT-PCR was 387 bp.

#### **3.3.4.3 PCR amplification of the *RuBisCo* gene region**

The *rbcLa* F and *rbcLa* R primer pair were used for amplification of the *RuBisCo* gene. The PCR programme used was identical to what is described above, with the exception that an annealing temperature of 55°C was used. Successful amplification of the *RuBisCo* gene regions results in the production of a 599 bp PCR product.

#### **3.3.5 Sensitivity of Soyblotch pss-RT-PCR**

To assess the sensitivity of the pss-RT-PCR, 10-fold serial dilutions of Soyblotch RT-PCR products were prepared. The initial starting concentration was determined with a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, California), according to the manufacturer's instructions. Second strand synthesis using the Soyblotch R-Tag primer, Exo I treatment and PCR amplification was performed as described above using the Soyblotch F and tag-specific primer pair (Soyblotch pss-RT-PCR), with the exception that primer and template were heated to 95°C and cooled to 4°C to denature double stranded template DNA before second strand synthesis.

### **3.4 Results**

#### **3.4.1 Specificity of Soyblotch pss-RT-PCR**

The specificity of the assay was tested on RNA extracts of two plant isolates, 16/4265 and 16/4266, which had tested positive for SbBMV using the diagnostic Soyblotch RT-PCR. RNA of both the genomic and anti-genomic strand should be present in infected plant material in which the virus is replicating. Reverse transcription of the positive sense RNA strand was primed by the Soyblotch R-Tag and the Soyblotch R primer, and cDNA transcribed from the Soyblotch F primer was used to confirm that PCR amplification of the antisense RNA strand did not occur.

### **3.4.1.1 Absence of detection of genomic RNA and misprimed cDNAs using the Soyblotch pss-RT-PCR**

cDNA synthesis using the Soyblotch F, unmodified Soyblotch R and no RT primer was performed to assess whether the assay non-specifically detects the negative RNA strand, and its ability to detect falsely-primed cDNAs. PCR amplification of cDNA not digested with Exo I transcribed from the Soyblotch F primer resulted in multiple bands corresponding to non-specific PCR amplification (Figure 3.1). A band of the size of the PCR product of the unmodified Soyblotch primer pair (354 bp) was also present, but following Exo I digestion of unincorporated Soyblotch F primers, a single band of approximately 200 bp was obtained. BLASTn analysis of the sequence of the 200 bp PCR product indicated cross reaction of primers with multiple plant chloroplast genomes (E-value 0.0) in the NCBI database. The Soyblotch pss-RT-PCR thus failed to amplify negative strand RNA when the Soyblotch pss-RT-PCR included an Exo I digestion following reverse transcription.

PCR products from amplification of cDNA produced from the Soyblotch R primer were present in the absence of Exo I treatment of cDNA (Figure 3.1). Unincorporated Soyblotch R primers from the reverse transcription reaction enabled amplification of a portion of the SbBMV polymerase gene as a result of the presence of both the Soyblotch F and Soyblotch R primer. This PCR product corresponds to the PCR product usually obtained from the unmodified Soyblotch primer pair. Sequence analysis of the PCR product also showed identity to a portion of the *L* gene of SbBMV (BLASTn E-values of 0.0 to EU 877231.1). However, following Exo I digestion of cDNA, this non-specific amplification was eliminated.

PCR amplification of cDNA transcribed in the absence of a primer using the Soyblotch RT-PCR yielded a band of 354 bp (Figure 3.2). Amplification using the Soyblotch F and tag-specific primers of the Soyblotch pss-RT-PCR was however better able to discriminate between falsely-primed cDNAs and cDNAs transcribed from the positive sense RNA strand, as no amplification occurred in the latter case. The low-level false-positive results observed

at lower annealing temperatures in this experiment may possibly be the result of contamination of reagents or the environment with Soyblotch R-Tag primer or PCR products of the Soyblotch pss-RT-PCR. The increased annealing temperature used in this experiment was not used in the standard Soyblotch pss-RT-PCR as this was balanced with an increased probability of obtaining false negative results.

#### **3.4.1.2 Specificity of Soyblotch pss-RT-PCR for positive sense strand**

PCR amplification of cDNA not subjected to Exo I digestion transcribed from the Soyblotch R-Tag primer yielded amplification in both 16/4265 and 16/4266 (Figure 3.1). In contrast, amplification was only observed for 16/4265 after unincorporated Soyblotch R-Tag primer from cDNA synthesis was removed by Exo I digestion. The sequence of the PCR product amplified from cDNA synthesis using the Soyblotch R-Tag primer showed significant homology (BLASTn E-value of 0.0) to the SbBMV polymerase sequence (EU 877231.1) in the NCBI database (<http://www.ncbi.nlm.nih.gov/>). Specific detection of the positive strand RNA of SbBMV thus relies on Exo I digestion of unincorporated primers to avoid false positive results.

#### **3.4.2 Sensitivity of Soyblotch pss-RT-PCR**

The sensitivity of the pss-RT-PCR was evaluated by performing cDNA synthesis using a dilution series of PCR products, ranging between 196 fg and 19.6 ag. The pss-RT-PCR was able to amplify template across the first five dilutions, and was thus able to detect the tagged, positive RNA strand of SbBMV across five orders of magnitude (Figure 3.3).

#### **3.4.3 Screening of SbBMV isolates using Soyblotch pss-RT-PCR**

PCR amplification using the RuBisCo-RT-PCR and Soyblotch RT-PCR was successful in all nine isolates tested, confirming RNA quality for RT-PCR analysis and presence of SbBMV in soybean plant material respectively (Figure 3.4). For all isolates except 95/0015 and 03/4033

the positive strand was successfully detected using the Soyblotch pss-RT-PCR, confirming the replication of the specific accessions in soybean plant material.

### **3.5. Discussion**

In this study, a pss-RT-PCR, enabling the detection of the positive sense, antigenomic RNA strand of SbBMV was developed. The use of a reverse primer tagged with a non-viral sequence during reverse transcription, combined with a tag-specific primer during PCR amplification allowed for the specific detection of the SbBMV replicative strand, eliminating false positives as a result of mis-priming. Exo I treatment of cDNA before PCR amplification further enhanced the specificity of the assay, preventing amplification as a result of false-priming and contaminating Soyblotch R-Tag RT primer in the PCR reaction.

Reports in literature of reverse transcription of cDNAs in the absence of primers illustrates how false-priming can occur (Timofeeva and Skrypina 2001; Peyrefitte et al. 2003). Priming of reverse transcription by the reverse transcriptase through secondary structures present in RNA or other short endogenous or exogenous sequences have been postulated as possible mechanisms facilitating false priming of cDNAs. Thus when unmodified or no reverse primers are used for cDNA synthesis, both specific and falsely-primed cDNAs can be amplified by virus specific primers in a PCR reaction.

This was observed when RNA of 16/4265 was subjected to reverse transcription in the absence of any primer. Subsequent PCR amplification of cDNA using the Soyblotch RT-PCR led to the amplification of the 354 bp portion of the SbBMV *L* gene against which the Soyblotch RT-PCR primer pair is directed. Due to the lack of primers in the reaction, the strand from which false-priming occurred cannot be determined, but it is likely that the genomic strand, which is more abundant, served as template (Plaskon et al. 2009). False-priming of cDNA synthesis in the absence of primer in the reaction has also been reported in o'nyong-nyong (ONNV), dengue and hepatitis viruses (Plaskon et al. 2009; Peyrefitte et al. 2003; Gunji et al. 1994; Lanford et al. 1994).

By using a non-viral tagged RT primer coupled with a tag-specific primer during PCR amplification, only virus-specific cDNAs in which the non-viral tag sequence was incorporated during reverse transcription should be detected, eliminating amplification of falsely-primed cDNAs. No amplification occurred when PCR of falsely-primed cDNAs (reverse transcription without primer) was performed with the Soyblotch F and tag-specific primers (Soyblotch pss-RT-PCR). This specificity was also illustrated by failure to amplify positive RNA strand-specific products from cDNA transcribed from the Soyblotch F and unmodified Soyblotch R products. Reverse transcription using the Soyblotch F primer detects the genomic sense RNA of SbBMV, and is used in the diagnostic Soyblotch RT-PCR, and thus served as negative control for the Soyblotch pss-RT-PCR. Failure to amplify the 387 bp PCR product of the Soyblotch pss-RT-PCR thus confirmed the specificity of the assay for the positive strand RNA.

However, failure to remove unincorporated RT primer can also lead to false positive results. Despite the improvements in the specificity of ss-RT-PCR as a result of the use of tagged primers and tag-specific primers, a number of other factors can still contribute towards non-specific amplification. This includes contamination of synthetic RNA transcripts with plasmid DNA, and contamination of PCR reactions with the primer used in reverse transcription (Craggs et al. 2001). The lack of strand-specificity observed after reverse transcription of 16/4266 with the Soyblotch R-Tag primer as illustrated by the absence of amplification following Exo I treatment was attributed to self-priming and contamination of the PCR reaction with RT primer (Purcell et al. 2006). The presence of the Soyblotch R-Tag primer in the PCR reaction containing Soyblotch F and tag-specific primers allowed amplification of mis-primed cDNA.

The importance of the removal of all free, unincorporated RT primer was also observed in the PCR amplification of cDNA transcribed from the Soyblotch R primer. In the absence of Exo I digestion of cDNA, contaminating Soyblotch R primer in the PCR reaction allowed amplification of the Soyblotch RT-PCR PCR product without the tag. Exo I digestion of

unincorporated primers, however, eliminated this false positive result of the slightly smaller PCR product. These results highlights the importance of combining Exo I digestion of cDNA before PCR amplification with tag-specific PCR primers to eliminate detection of false positive results.

After establishment of this protocol, nine isolates of SbBMV from five different locations in South Africa sampled over 12 years were selected for screening using the Soyblotch pss-RT-PCR. An internal plant barcoding gene was first amplified to ensure RNA integrity and the presence of SbBMV confirmed before performing the Soyblotch pss-RT-PCR. In seven out of the nine isolates, the positive strand of SbBMV was detected. The negative results in two isolates were unexpected, and might be due to a recovery phenotype and plant defence responses or simply tissue selection during the RNA extraction. The recovery of tobacco plants from symptoms induced by Tobacco ringspot virus was the first report of a recovery phenotype in plants infected by plant viruses (Wingard 1928). Symptoms decline due to a decrease in virus titre, but the virus can still be present in recovered leaves, which are also often resistant to subsequent infections (Ghoshal and Sanfaçon 2015). This, however, further illustrates the specificity of the assay, as the positive sense RNA was not detected in all isolates positive for the presence of the virus.

In this study we described the development of a pss-RT-PCR to detect the replicative intermediate of SbBMV. The assay is capable of detecting the positive RNA strand of SbBMV specifically over five orders of magnitude, and will be used in future to detect the intermediate replicative RNA strand of SbBMV in its insect vector *P. carboverdensis*. An enhanced understanding of the replication dynamics of SbBMV in its vector will improve control strategies against the virus in future.

### **3.6. Acknowledgements**

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## Tables

**Table 3.1. Nucleotide sequences (5'-3') of oligonucleotide primers used in this study.**

| Primer              | Sequence                                         |
|---------------------|--------------------------------------------------|
| rbclLa F            | ATGTCACCACAAACAGAGACTAAAGC                       |
| rbclLa R            | GTAAAATCAAGTCCACCRCC                             |
| Soyblotch F         | CTTTGCCCAACTGGACTCCC                             |
| Soyblotch R         | TCCAAACAGTCTTCCCAGGC                             |
| Soyblotch R-Tag     | <u>GGCCGTCATGGTGGCGAATA</u> TCCAAACAGTCTTCCCAGGC |
| Tag-specific primer | AATAAATCATAAGGCCGTCATGGTGGCGAATAA                |

The non-viral tag sequence on the Soyblotch R-Tag primer is underlined.

**Table 3.2. Host, geographical origin, year of collection and accession information of SbBMV isolates used in this study.**

| Accession | Host               | Origin                                             | Year |
|-----------|--------------------|----------------------------------------------------|------|
| 15/3079   | <i>Glycine max</i> | Loskop Irrigation Scheme, Mpumalanga, South Africa | 2015 |
| 15/3080   | <i>Glycine max</i> | Loskop Irrigation Scheme, Mpumalanga, South Africa | 2015 |
| 15/3083   | <i>Glycine max</i> | Loskop Irrigation Scheme, Mpumalanga, South Africa | 2015 |
| 16/4265   | <i>Glycine max</i> | Brits, North West, South Africa                    | 2016 |
| 16/4266   | <i>Glycine max</i> | Brits, North West, South Africa                    | 2016 |
| 17/5002   | <i>Glycine max</i> | Brits, North West, South Africa                    | 2017 |
| 95/0015   | <i>Glycine max</i> | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1995 |
| 95/0038   | <i>Glycine max</i> | Thabazimbi, Limpopo, South Africa                  | 1995 |
| 95/0073   | <i>Glycine max</i> | Pretoria, Gauteng, South Africa                    | 1995 |
| 03/4025   | <i>Glycine max</i> | Lusikisiki, Eastern Cape, South Africa             | 2003 |
| 03/4033   | <i>Glycine max</i> | Lusikisiki, Eastern Cape, South Africa             | 2003 |

## Figures

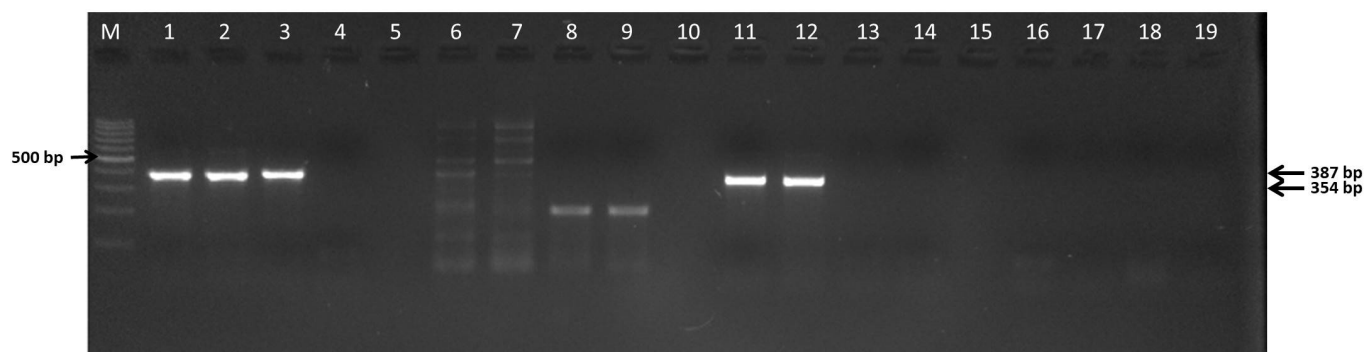


**Figure 3.1.** Impact of the primer used for cDNA transcription and Exonuclease I (Exo-I) treatment on the specificity of the Soyblotch pss-RT-PCR assay. **M**, 100 bp DNA ladder; **1**, 16/4265 Soyblotch R-Tag; **2**, 16/4266 Soyblotch R-Tag; **3**, 16/4265 Soyblotch R-Tag digested with Exo I following cDNA synthesis; **4**, 16/4266 Soyblotch R-Tag digested with Exo I following cDNA synthesis; **5**, unused lane; **6**, 16/4265 Soyblotch F; **7**, 16/4266 Soyblotch F; **8**, 16/4265 Soyblotch F digested with Exo I following cDNA synthesis; **9**, 16/4266 Soyblotch F digested with Exo I following cDNA synthesis; **10**, unused lane; **11**, 16/4265 Soyblotch R; **12**, 16/4266 Soyblotch R; **13**, 16/4265 Soyblotch R digested with Exo I following cDNA synthesis; **14**, 16/4266 Soyblotch R digested with Exo I following cDNA synthesis; **15**, unused lane; **16**, Soyblotch R-Tag no template cDNA control; **17**, Soyblotch F no template cDNA control; **18**, Soyblotch R no template cDNA control, **19**, no template PCR control.

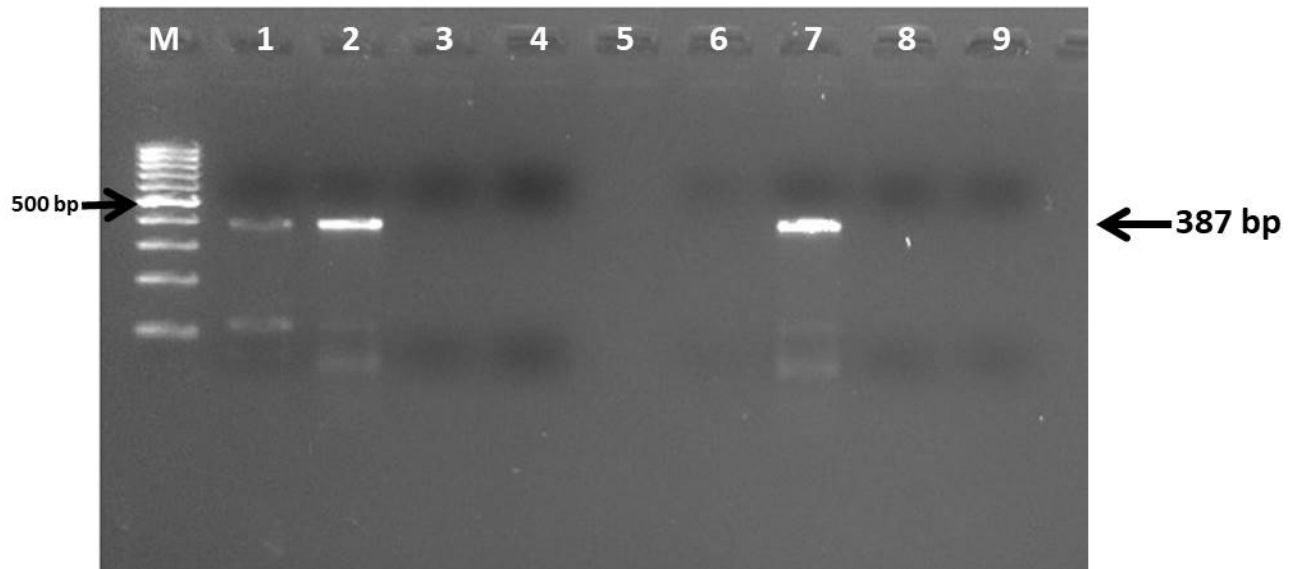


**Figure 3.2.** Specificity analysis of pss-RT-PCR using a tag-specific primer for PCR amplification. Lanes 1-4 represent PCR amplification using the Soyblotch F and Soyblotch R primer pair, and lanes 6-9 PCR amplification using the Soyblotch F and tag-specific primer. **M**, 100 bp DNA ladder; **1**, 16/4265 reverse transcription in absence of primer; **2**, 16/4265 reverse transcription with Soyblotch R-Tag primer; **3**, no template cDNA synthesis control; **4** no template PCR control; **5**, unused lane; **6**, 16/4265 reverse transcription in absence of primer; **7**, 16/4265 reverse transcription with Soyblotch R-Tag primer; **8**, no template cDNA synthesis control; **9**, no template PCR control.

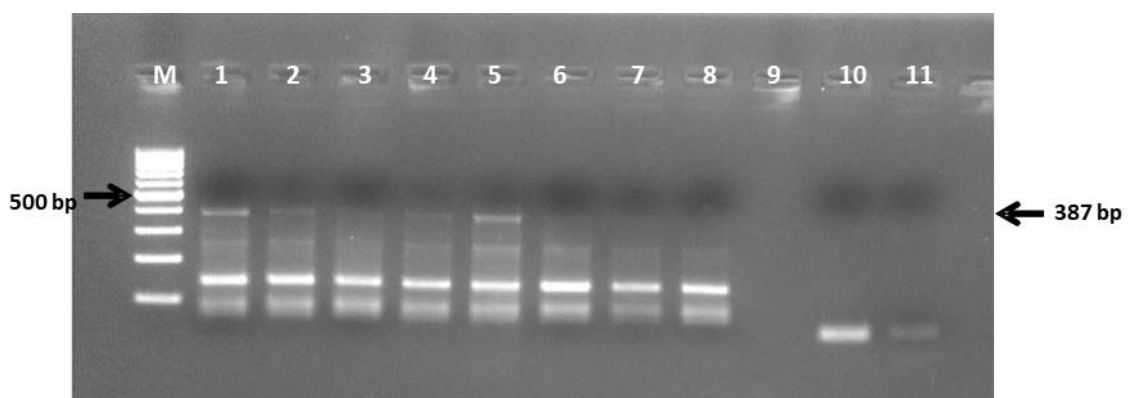


**Figure 3.3.** Sensitivity analysis of pss-RT-PCR for SbBMV. **M**, 100 bp DNA ladder; **1**, 196 pg; **2**, 19.6 pg; **3**, 1.96 pg; **4**, 196 fg; **5**, 19.6 fg; **6**, 1.96 fg; **7**, 196 ag; **8**, 19.6 ag; **9**, unused lane; **10**, no template cDNA synthesis control; **11**, no template PCR control.

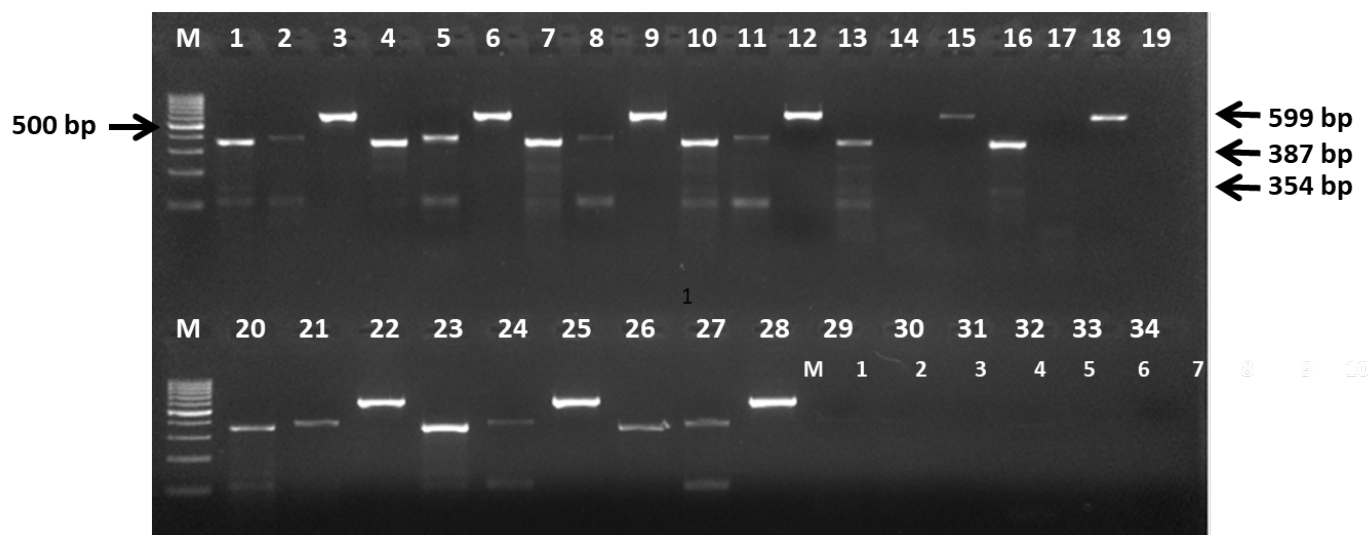


**Figure 3.4.** Analysis of field samples using the pss-RT-PCR. **M**, 100 bp DNA ladder; **1**, 15/3083 Soyblotch RT-PCR product; **2**, 15/3083 pss-RT-PCR product; **3**, 15/3083 *rbcLa* PCR product; **4**, 15/3080 Soyblotch RT-PCR product; **5**, 15/3080 pss-RT-PCR product; **6**, 15/3080 *rbcLa* PCR product; **7**, 95/0038 Soyblotch RT-PCR product; **8**, 95/0038 pss-RT-PCR product; **9**, 95/0038 *rbcLa* PCR product; **10**, 17/5002 Soyblotch RT-PCR product; **11**, 17/5002 pss-RT-PCR product; **12**, 17/5002 *rbcLa* PCR product; **13**, 95/0015 Soyblotch RT-PCR product; **14**, 95/0015 pss-RT-PCR product; **15**, 95/0015 *rbcLa* PCR product; **16**, 03/4033 Soyblotch RT-PCR product; **17**, 03/4033 pss-RT-PCR product; **18**, 03/4033 *rbcLa* PCR product; **19**, unused lane; **20**, 15/3079 Soyblotch RT-PCR product; **21**, 15/3079 pss-RT-PCR product; **22**, 15/3079 *rbcLa* PCR product; **23**, 95/0073 Soyblotch RT-PCR product; **24**, 95/0073 pss-RT-PCR product; **25**, 95/0073 *rbcLa* PCR product; **26**, 03/4025 Soyblotch RT-PCR product; **27**, 03/4025 pss-RT-PCR product; **28**, 03/4025 *rbcLa* PCR product; **29**, Soyblotch RT-PCR no template cDNA synthesis control; **30**, pss-RT-PCR no template cDNA synthesis control; **31**, *rbcLa* RT-PCR no template cDNA synthesis control; **32**, Soyblotch RT-PCR no template PCR control; **33**, pss-RT-PCR no template PCR control; **34**, *rbcLa* no template PCR control.

## **Chapter 4**

**Transmission of Soybean blotchy mosaic  
virus by the leafhopper, *Peragallia  
caboverdensis* Lindberg (*Hemiptera:  
Cicadellidae: Megophthalminae*)**

#### 4.1 Abstract

Soybean blotchy mosaic virus (SbBMV) is a significant yield-limiting virus of soybean production in the warmer, lower-lying regions of South Africa. The annual re-introduction of SbBMV onto soybean results in the persistence of disease across soybean seasons. In this study, the role of *Peragallia caboverdensis* Lindberg 1956, the leafhopper vector of SbBMV, in the seasonal persistence of SbBMV was investigated. A survey over 13 months for *P. caboverdensis* was conducted in close proximity to areas where high incidences of symptomatic soybean was present to determine its incidence and prevalence. All *P. caboverdensis* individuals collected were tested for the presence of SbBMV by RT-PCR, and SbBMV-positive accessions also screened using a positive strand-specific-RT-PCR (pss-RT-PCR) to detect replication of SbBMV in *P. caboverdensis*. The nucleotide sequence of two insect barcoding genes, mitochondrial cytochrome c oxidase subunit I (*mtCOI*) and histone 3 (*H3*) were also determined to enable identification of *P. caboverdensis* using molecular techniques in future. A total of 754 *P. caboverdensis* accessions were collected over 13 months, of which 24 tested positive for SbBMV. A permanent, year-round presence for *P. caboverdensis* was confirmed, and SbBMV-positive *P. caboverdensis* accessions were detected in both the soybean production and off-seasons. Replication of SbBMV in *P. caboverdensis* was not detected in the 24 SbBMV-positive accessions subjected to the pss-RT-PCR. Partial nucleotide sequences for the *mtCOI* and *H3* genes were determined, and the generation of *mtCOI* and *H3* nucleotide sequences of closely related leafhopper species and additional *P. caboverdensis* individuals from other geographic locations will confirm if successful species delineation is possible using these molecular markers. Monitoring of viral load in a viruliferous colony by RT-qPCR or the pss-RT-PCR can provide conclusive evidence for the mode of transmission of SbBMV by *P. caboverdensis* in future.

## 4.2 Introduction

Soybean blotchy mosaic virus (SbBMV) is a member of the genus *Cytorhabdovirus* in the family *Rhabdoviridae* (Lamprecht et al. 2010). Rhabdoviruses have linear, single-stranded, negative sense RNA genomes (non-segmented or bipartite), with or without a host-derived lipoprotein membrane (Amarasinghe et al. 2017). Plant rhabdovirus particles are bacilliform in shape, and the 12 to 14.5 kB genomic RNA encodes five conserved proteins, the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase (L) (Dietzgen et al. 2017; Dietzgen and Kuzmin 2012). Plant rhabdoviruses in the genera *Nucleo*- and *Cytorhabdovirus* also encode accessory proteins involved in the spread of virions throughout the plant (Walker et al. 2011). The host range of rhabdoviruses includes reptiles, mammals, fish and plants (Jackson et al. 2005), and some members of the family *Rhabdoviridae*, including the plant-infecting genera *Nucleorhabdovirus* and *Cytorhabdovirus* also replicate in their invertebrate vectors, implicating the presence of more than one natural host for some species.

Plant rhabdoviruses generally exhibit a high degree of vector specificity, where one vector species would be responsible for the transmission of a single virus. As an example, five maize-infecting viruses, Maize mosaic virus (MMV), Maize fine streak virus (MFSV), Wheat american striate mosaic virus, Maize iranian mosaic virus and Sorghum stunt mosaic virus are all transmitted by only six different vector species (Jackson et al. 2005). This close evolutionary relationship probably allows for the persistent, replicative transmission of plant rhabdoviruses by their insect vectors. Replication in their respective insect vectors have been reported for the plant rhabdoviruses MMV, Northern cereal mosaic virus, Barley yellow striate virus, MFSV, Rice yellow stunt virus and Wheat chlorotic streak virus (Barandoc-Alviar et al. 2016; Whitfield et al. 2015; Ammar and Hogenhout 2008; Conti 1985; Ammar and Nault 1985; Falk and Tsai 1985; Todd et al. 2010).

SbBMV infection of soybean results in the development of distinctive blotchy mosaic symptoms on leaf material. Disease incidences as high as 32% have been observed, which resulted in yield losses up to 20% (Pietersen 1993). The disease has been reported from warmer, lower-lying soybean production areas of South Africa such as the North West, Limpopo, Mpumalanga, Gauteng, Eastern Cape and KwaZulu-Natal provinces (Strydom and Pietersen 2018c; Pietersen and Garnett 1990; Pietersen et al. 1998; Lamprecht et al. 2010). Soybean is commonly grown in rotation with winter grains such as wheat in these areas in South Africa, but symptoms re-appear early in each new season, and often decline as the season progresses.

A number of mechanisms can facilitate the re-emergence of disease on soybean in each new season. Alternative plant hosts of SbBMV, which can act as viral reservoirs during the soybean off-season, have been identified (Strydom and Pietersen 2018a). The appearance of symptoms early in the soybean season might be attributed to seasonal persistence of SbBMV through infected seed, but seed transmissibility of SbBMV was not detected in four commercial soybean cultivars tested (Strydom and Pietersen 2018a). No evidence for seed transmission has been found in any of the rhabdoviruses (Jackson et al. 2005). Finally, persistent, propagative and/or vertical transmission of SbBMV by its insect vectors can also allow for the re-introduction of the virus onto soybean each year.

The leafhopper *Peragallia caboverdensis* Lindberg (*Cicadellidae: Megophthalminae: Agalliini* (previously erroneously as *Agallinae* in Lamprecht et al. 2010; Strydom and Pietersen 2018a, 2018c) has been shown to transmit SbBMV to soybean (Lamprecht et al. 2010). It was able to transmit SbBMV from infected, unrooted cuttings to healthy soybean plants through two serial passages, after which they lost their infectivity. Following the first report of *P. caboverdensis* in the Cape Verde Islands, their geographic distribution has expanded to include Morocco, Aldabra Islands, Madeira, Mauritius, Zimbabwe and Salvages (Lindberg 1965; Webb 1980; Quartau and Andre 1980; Lindberg 1985). Very little ecological,

morphological and taxonomic information for *P. caboverdensis* is present in literature, and no molecular sequence data has been generated to date

In this study, a 13 month survey for *P. caboverdensis* was conducted in close proximity to areas where soybean displaying high incidences of the characteristic blotchy mosaic symptoms associated with SbBMV infection was present. Accessions of this leafhopper positive for SbBMV were identified by RT-PCR to establish whether they could be detected across all seasons. Accessions positive for SbBMV were also subjected to a positive strand-specific-RT-PCR (pss-RT-PCR) to determine whether SbBMV replicates in this leafhopper as reported for other plant rhabdoviruses. Finally, two barcoding genes, mitochondrial cytochrome *c* oxidase subunit I (*mtCOI*) and histone 3 (*H3*) (nuclear) were amplified by RT-PCR and sequenced to aid identification of *P. caboverdensis* in future.

### **4.3 Materials and Methods**

#### **4.3.1 Survey for leafhoppers in a soybean production area with a high SbBMV incidence**

From April 2017 to April 2018 ten yellow sticky insect traps were placed in close proximity to an area where soybeans displaying high incidences of SbBMV were present since at least 2014 in Brits, North West, South Africa. Sticky traps were collected from the field after one week, and leafhoppers removed from the traps by using chloroform to dissolve the glue. Leafhoppers were rinsed in chloroform to remove residual glue, and stored in 100% ethanol until processing.

#### **4.3.2 Identification of leafhoppers based on morphology**

All leafhoppers collected were identified by either the first or second author. Leafhoppers were identified by their morphology, and identification to species level was performed on representative specimens by removing the last few segments of the abdomen, followed by soaking in cold KOH until clear and examination of sex organs using a stereo microscope

(Quartau and Andre 1980). Voucher specimens with accession numbers CCDL27772 (18/6587) and CCDL27773 (18/6588) were deposited in the National Collection of Insects, Agricultural Research Council, Pretoria.

#### **4.3.3 Non-destructive DNA extraction from voucher specimens**

DNA was purified from two voucher specimens (CCDL27772 (18/6587) and CCDL27773 (18/6588)) by incubating specimens in digestion buffer overnight as described by Thomsen et al. (2009), followed by DNA extraction using a modified CTAB method described previously with a few modifications (Strydom and Pietersen 2018a, 2018c). Briefly, insects were allowed to dry following removal from 100% ethanol, placed in 50 µl digestion buffer (3 mM CaCl<sub>2</sub>, 2% sodium dodecyl sulphate (SDS), 40 mM dithiotreitol (DTT), 250 mg/ml proteinase K, 100 mM Tris buffer pH 8 and 100 mM NaCl) (Thomsen et al. 2009) and incubated overnight at 55°C with gentle shaking (100 rpm). Specimens were removed from the digestion buffer and replaced in 100% ethanol, and nucleic acids extracted from the digestion buffer as described previously with some modifications. The CTAB and digestion buffer were incubated at 65°C for 15 min, followed by one C:I extraction. DNA was precipitated through the addition of two volumes of 100% ethanol, and incubation at -20°C for 1 hour.

#### **4.3.4 RNA extraction from leafhoppers**

RNA was extracted from individual leafhoppers using Trizol reagent (Life Technologies, Carlsbad, USA). Each leafhopper was macerated in 150 µl Trizol reagent using a mortar and pestle, and incubated for 30 min at room temperature to allow for complete nucleoprotein dissociation. This was followed by the addition of 30 µl chloroform, incubation for 2 min at room temperature and centrifugation at 18928 xg for 15 min at 4°C. The supernatant was transferred to a new tube, an equal volume of isopropanol was added and tubes were incubated at room temperature for 30 min. Nucleic acids were precipitated by centrifugation at 18928 xg for 30 min at 4°C. The supernatant was discarded and 500 µl 75% ethanol

added, followed by centrifugation at 1136  $\times g$  for 15 min at 4°C. Ethanol was removed, followed by a final centrifugation step at 1136  $\times g$  for 5 min to remove residual droplets of ethanol. Pellets were air-dried at room temperature, and re-suspended in 20  $\mu$ l molecular grade H<sub>2</sub>O.

#### **4.3.5 Detection of SbBMV in *P. caboverdensis* using RT-PCR**

RT-PCR and pss-RT-PCR was performed to detect the presence and replication of SbBMV in *P. caboverdensis* respectively as described previously (Strydom and Pietersen 2018b). The diagnostic Soyblotch RT-PCR utilizes the Soyblotch F and Soyblotch R primers (Table 4.1) to amplify a 354 bp region of the SbBMV *L* gene available on the National Centre for Biotechnology Information database (NCBI) (EU 877231.1) (Strydom and Pietersen 2018a). Individual leafhoppers which tested positive for the presence of SbBMV were also subjected to the Soyblotch pss-RT-PCR (Strydom and Pietersen 2018b) to determine whether replication of SbBMV could be detected in *P. caboverdensis*. The pss-RT-PCR relies on the use of the Soyblotch R-Tag primer (Table 4.1) during reverse transcription to specifically detect SbBMV positive sense RNAs corresponding to the SbBMV *L* gene, followed by PCR amplification using the Soyblotch F and tag-specific R primers (Table 4.1).

#### **4.3.6 RT-PCR amplification and Sanger sequencing of *COI* and *H3* in *P. caboverdensis***

Partial *COI* and *H3* gene regions of 25 *P. caboverdensis* accessions were amplified by PCR using the LCO (Folmer et al. 1994) and Hex (Ogden and Whiting 2003) primer pairs respectively, and sequences determined by Sanger sequencing. For synthesis of the complementary strand, 2  $\mu$ l RNA and 5  $\mu$ l random hexamer primer (4  $\mu$ M primer in final reaction) were allowed to anneal by heating to 70°C for 5 min, followed by cooling at 4°C for 5 min. cDNA synthesis reactions (12.5  $\mu$ l) consisted of 7  $\mu$ l primer-RNA, 2.5  $\mu$ l M-MLV Reverse Transcriptase 5x Reaction buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT in final working solution) (Promega, Madison, USA), 1.25  $\mu$ L dNTPs (10 mM each dATP, dCTP, dGTP and dTTP) (Kapa Biosystems, Wilmington, USA), 0.125  $\mu$ l (25

U) M-MLV Reverse Transcriptase (Promega) and 1.6 µl molecular grade H<sub>2</sub>O. Reactions were incubated at 37°C for 60 min. Each PCR amplification reaction (25 µl) consisted of 2 µl cDNA, 2.5 µl 10x NH<sub>4</sub> Reaction Buffer (Bioline, London, UK), 2 µl MgCl<sub>2</sub> solution (50 mM) (Bioline), 0.75 µl dNTPs (10 mM each dATP, dCTP, dGTP and dTTP) (Kapa Biosystems) 0.5 µl (0.2 µM) forward primer (LCO1490 or Hex-AF) (Table 4.1), 0.5 µl (0.2 µM) reverse primer (LCO2198 or Hex-AR) (Table 4.1), 0.4 µl (2 U) BioTaq DNA polymerase (Bioline) and 16.35 µl molecular grade H<sub>2</sub>O. PCR cycling conditions consisted of an initial denaturation step at 95°C for 1 min, followed by 35 cycles of 95°C for 30 sec, 42°C for 30 sec and 72°C for 30 sec, with final extension of PCR products at 72°C for 5 min. Standard PCR using the LCO and H3 primer pairs was used to amplify the *COI* and *H3* genes from DNA of the two voucher specimens. Ethidium bromide-stained 2% agarose gels were used to separate PCR products (6 µl) by agarose gel electrophoresis, and viewed under UV light. For purification of PCR products prior to sequencing, 0.5 µl (10 U) Exo I (Thermo Scientific, Waltham, USA) and 2 µl (2 U) FastAP Alkaline Phosphatase (Thermo Scientific) was added to each 19 µl PCR product. This was followed by incubation at 37°C for 15 min, followed by incubation at 85°C for 15 min. Sequencing reactions (10 µL) consisted of 1 µl template DNA, 1 µl of BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Foster City, USA), 2.25 µl 5x Sequencing buffer (Applied Biosystems) and 2 µM of each primer. DNA in sequencing reactions was precipitated using standard ethanol precipitation. Sanger sequencing was conducted at the University of Pretoria, South Africa, using an ABI 3500xL automated sequencer.

#### **4.3.7 Phylogenetic analysis**

BioEdit software (Hall 1999) was used to assemble forward and reverse raw sequence reads. Consensus sequences were manually curated and aligned online with default parameters using Mafft (Kato et al. 2002). The influence of gaps present in alignments was minimised by trimming alignments from both the 5' and 3' ends in BioEdit. Phylogenetic trees were constructed using the neighbour-joining (NJ) method (Saitou and Nei 1987) in MEGA

software version 7 (Tamura et al. 2011) with 1000 replicates of bootstrap analysis. The common brown leafhopper, *Orosius orientalis* (COI: KR030339.1; H3: JX433639.1) was used as outgroup. Sequences obtained were subjected to Basic Local Alignment Search (BLASTn) analysis in NCBI.

#### **4.3.8 Statistical analysis**

Statistical analysis of data obtained during this survey for *P. caboverdensis* in Brits was performed using R software (R Core Team 2013). The standard deviation of each data point from the population mean was calculated, followed by analysis of variance (ANOVA). Tukey's Honest Significant Differences (HSD) test at a significance level of  $p < 0.05$  was used to identify significant differences between means.

### **4.4 Results**

#### **4.4.1 Leafhoppers identified during survey**

A number of leafhopper species were identified by morphology from the soybean production area displaying high SbBMV incidences in Brits, North West, South Africa (Table 4.2). Ten common species of leafhopper representing nine genera in three sub-families were identified, of which *P. caboverdensis* (Figure 4.1) was the dominant species collected. Previous studies identified *P. caboverdensis* as an insect vector of SbBMV, thus detection of SbBMV in leafhoppers using molecular techniques focussed on *P. caboverdensis* in this study. However, in future a role for other species of leafhopper in the transmission of SbBMV should be investigated.

#### **4.4.2 Detection of SbBMV in *P. caboverdensis***

The survey for *P. caboverdensis* confirmed the presence of SbBMV-positive *P. caboverdensis* accessions throughout the year. A total of 754 individuals of *P. caboverdensis* were obtained over 13 months, but no trend in the number of *P. caboverdensis* accessions collected was observed as the number of individuals collected

was significantly different for each month (Figure 4.2). During May 2017 to November 2017, 52.5% (396 specimens) of *P. caboverdensis* were collected, which generally corresponded to the soybean off-season. Additionally, the highest number of *P. caboverdensis* individuals obtained in one month was 197 in July 2017, demonstrating that *P. caboverdensis* successfully colonized alternative hosts in the absence of soybean in the winter months.

All 754 *P. caboverdensis* accessions collected were subjected to the diagnostic Soyblotch RT-PCR, of which 24 (3.2%) tested positive for SbBMV (Figure 4.2). As with the number of *P. caboverdensis* individuals collected on sticky traps, there was no trend in the number of SbBMV-positive *P. caboverdensis* accessions. Differences in the proportion of SbBMV-positive *P. caboverdensis* were observed in successive months, with no correlations to specific seasons. During May–June 2017, August–September 2017, and January–April 2018 no *P. caboverdensis* accessions tested positive for the presence of SbBMV. In contrast, SbBMV was detected in *P. caboverdensis* in 2017 in April (10 specimens), July (6), October (1), November (1) and December (2), and 2018 in February (2) and March (2). No positive results were obtained when SbBMV-positive accessions (24) were screened using the Soyblotch pss-RT-PCR, thus replication of SbBMV in *P. caboverdensis* was not detected.

#### **4.4.3 Phylogenetic analysis and barcoding of *P. caboverdensis***

Phylogenetic analysis of 538 bp of the *COI* (Figure 4.3) and 285 bp of the *H3* (Figure 4.4) genes respectively of 25 *P. caboverdensis* accessions (Table 4.3) using the NJ method is presented. Phylogenetic trees produced from sequence data of the *COI* and *H3* gene regions were similar in grouping all accessions except Accession 18/6266 together in a single clade with high bootstrap support. This confirmed that low intraspecific variation was present in the *COI* and *H3* genes of *P. caboverdensis*. The average mean pairwise distance in *COI* between accessions was 0.015, with a high of 0.178. For the *H3* gene region, the maximum pairwise distance was 0.009, with a mean of 0.098. BLASTn analysis of sequence data corresponding to the *COI* gene identified *Dryodurgades lamellaris* (KU 642027.1) as the

closest relative of all *P. caboverdensis* accessions except accession 18/6266 in the NCBI database. The most significant hit obtained for the partial *COI* gene of accession 18/6266 was *Cicada* spp. (LN 879021.1). For the *H3* gene region, all *P. caboverdensis* accessions except 18/6266 showed the most significant BLASTn homology to *Bemisia tabaci* (XM 019048657.1). The *H3* sequence of accession 18/6266 was most closely related to *Bulacta exarta* (HQ 834193.1).

#### 4.5 Discussion

The role of *P. caboverdensis* in the seasonal persistence of SbBMV was investigated for the first time during this study. Previous studies identified *P. caboverdensis* as a vector of SbBMV following successful transmission of SbBMV to healthy soybean plants, but the exact mode of transmission was not determined (Lamprecht et al. 2010). This study reports on the incidence and prevalence of *P. caboverdensis* in a SbBMV-affected area in both soybean production and off-seasons for the first time. A year-round presence and association of *P. caboverdensis* with SbBMV was identified by RT-PCR, but replication of SbBMV in *P. caboverdensis* was not detected. With the determination of the partial gene sequences of two insect barcoding genes, *COI* and *H3* the first molecular sequence data for *P. caboverdensis* was generated. This will enable identification of *P. caboverdensis* using molecular characters in future, and contribute towards further studies aimed at resolving the taxonomy of leafhoppers.

The re-introduction of SbBMV onto soybean each year appears to occur by two mechanisms, both of which are facilitated by *P. caboverdensis*. Feeding of *P. caboverdensis* on SbBMV-infected alternative plant hosts prior to feeding on healthy soybean can result in the introduction and subsequent spread of SbBMV on soybean. Successful infection of annual and perennial alternative plant hosts by SbBMV in both the summer and winter months have been reported (Strydom and Pietersen 2018a). This implies that SbBMV may over-winter on alternative hosts in the absence of soybean, followed by re-introduction of

SbBMV onto soybean by *P. caboverdensis* in the new production season. Secondly, transmission of SbBMV by *P. caboverdensis* in a persistent, circulative or persistent, propagative manner can allow SbBMV to bridge the gap between the soybean production and off-seasons in *P. caboverdensis* without a requirement for SbBMV-reservoirs.

A year-round presence of *P. caboverdensis* in an area displaying high incidences of SbBMV annually for at least four consecutive years was shown with the 13-month survey for *P. caboverdensis*. Individuals of this leafhopper occurred throughout the summer and winter months, and the apparent stable population cycle can indicate the presence of a large population of *P. caboverdensis* consisting of individuals at different life stages as a result of the longterm presence of *P. caboverdensis* in the area. Interestingly, the highest number of individuals collected in a single month was in July 2017, confirming the successful colonization of other plant hosts and the strong presence of SbBMV in the soybean off-season. This was also evident in the detection of eight SbBMV-positive *P. caboverdensis* accessions in the period May 2017-November 2017, when soybean was absent. These *P. caboverdensis* accessions could have acquired SbBMV through feeding on alternative hosts or may have maintained infection as a result of persistent or propagative transmission. The detection of *P. caboverdensis* individuals positive for SbBMV throughout the year shows that the presence of alternative plant hosts and/or transmission in a persistent manner by *P. caboverdensis* facilitates the persistence of SbBMV on soybean.

The failure to detect replication of SbBMV in *P. caboverdensis* in this study, together with previous reports of SbBMV only transmitted to soybean through two serial passages, after which SbBMV was no longer transmitted, suggests that SbBMV may not replicate actively in all leafhopper life stages or that it may not be transmitted in a persistent, propagative manner as reported for other plant rhabdoviruses (Barandoc-Alviar et al. 2016; Whitfield et al. 2015; Ammar and Hogenhout 2008; Conti 1985; Ammar and Nault 1985; Falk and Tsai 1985; Todd et al. 2010). Most plant viruses transmitted by planthoppers and leafhoppers are transmitted persistently (Nault and Rodriguez 1985; Nault 1997), thus *P. caboverdensis* may

be transmitted persistently in only a circulative, and not a propagative, manner. The presence of SbBMV-reservoirs in the form of alternative hosts may also contribute to the maintenance of viral load in *P. caboverdensis* if it transmits SbBMV in a persistent, circulative manner. However, as persistent, propagative transmission seems to be conserved in those plant nucleo- and cytorhabdoviruses in which transmission dynamics have been investigated (Barandoc-Alviar et al. 2016; Whitfield et al. 2015; Ammar and Hogenhout 2008; Conti 1985; Ammar and Nault 1985; Falk and Tsai 1985; Todd et al. 2010), the presence of additional *P. caboverdensis* vectors should be investigated.

Although the presence of multiple vectors for plant rhabdoviruses is rare, plant viruses transmitted by leafhoppers, planthoppers and aphids are often transmitted by vector species in the same family or sub-family (Nault 1997; Dietzgen and Kuzmin 2012). To date, only one vector species, *P. caboverdensis*, has been identified for SbBMV (Lamprecht et al. 2010). However, in the study in which *P. caboverdensis* was shown to transmit SbBMV, six other leafhopper morphogroups were also collected but not subjected to transmission experiments. This highlights the importance of investigating possible roles in the transmission of SbBMV for other leafhopper species identified in this study, as an association with soybean was already shown. Their ability to colonize soybean and alternative plant hosts, together with a capacity to transmit SbBMV should be investigated. When multiple vectors for a virus exists, one species usually acts as the primary vector, transmitting the virus more efficiently than other, secondary vector species (Hibino 1996; Sinha 1970). Additional SbBMV vectors may thus transmit SbBMV in the persistent, propagative manner widely reported in the plant rhabdoviruses.

The failure to detect SbBMV replication in *P. caboverdensis* in this study could also be the result of the sampling strategy and resulting relatively small sampling size. The survey did not select for SbBMV-positive individuals, and thus only 3.2% of *P. caboverdensis* accessions collected were positive for SbBMV. It would be expected that only a fraction of individuals collected in this manner would exhibit replication of SbBMV if this was to occur,

as factors such as optimal acquisition times, latent periods etc. cannot be controlled. In future, the establishment of a *P. caboverdensis* colony, uniform in age, will be an important tool to further investigate the transmission dynamics of SbBMV in *P. caboverdensis*. Viral load in *P. caboverdensis* individuals transferred from SbBMV-positive soybean to healthy soybean can be monitored over time in order to determine whether viral load decreases, remains constant or increases. Alternatively, purified virions can be fed to the leafhopper through a membrane (Rochow 1960). The Soyblotch pss-RT-PCR used in this study can also be employed more efficiently on a colony of *P. caboverdensis* feeding on SbBMV-positive soybean.

The *COI* gene region is the most widely used molecular character in the DNA barcoding of insects (and animals) as a result of the high interspecific and low intraspecific sequence diversity between and within taxa respectively (Hebert et al. 2003a; Hebert et al. 2003b; Hebert and Gregory 2005). It has been employed in other groups of the *Hemiptera* such as *Heteroptera*, *Coccoidea*, *Aphididae* and *Adelgidae*, but barcoding sequence data for the hoppers remain limited (Lin et al. 2004). Despite low interspecific distances reported for some species in the sub-order *Auchenorrhyncha* (plant-, leaf- and treehoppers) (Footitt et al. 2014), *COI* has successfully been employed in delineating others (Sreejith and Sebastian 2015; Cryan and Svenson 2010; Bluemel et al. 2011). The nuclear *H3* gene has also proved to be a useful molecular marker in phylogenetic studies in the leafhoppers, resulting in phylogenies consistent with *COI*, biogeographic and morphological data (Meshram et al. 2017; Wang et al. 2017), and can be used in conjunction with *COI* to confirm species identifications.

The phylogenies produced from the *COI* and *H3* gene sequences grouped all accessions except accession 18/6266 in a single clade with high bootstrap support. For the number and geographical origin of accessions analysed in this study, the *COI* and *H3* gene regions appear to exhibit sufficient levels of sequence conservation to allow for accurate species identification. In future, partial *COI* and *H3* gene sequences of *P. caboverdensis* accessions

from other geographical locations, as well as closely related species should also be determined to confirm successful species delimitation using these two gene regions. This will contribute towards understanding whether the individual belonging to accession 18/6266 belongs to another closely related species or if higher levels of sequence diversity are present in some individuals of the species. At the same time, the limited amount of DNA sequence information available for the leafhoppers complicates accurate species identification and taxonomy of the group, as seen from some of the most significant BLASTn matches of sequences generated in this study. The absence of DNA barcodes for 98% of insect species in NCBI results in frequent Type II errors, which result in misidentification when reference sequences are not present (Virgilio et al. 2010).

The generation of molecular sequence data for *P. caboverdensis* will aid fast and accurate identification of all life stages of *P. caboverdensis*. Morphological identification of *P. caboverdensis* focuses on male genitalia with colour patterns, which are difficult to discern. This problem is exasperated further through the occurrence of morphologically similar leafhopper species with common geographical ranges. The colouring of *Austragallia* spp collected in this study, for example is very similar to that found in *P. caboverdensis*. The sequence of the 28S rDNA gene region can also be determined, as it has been shown to be phylogenetically more informative than the *H3* region in some species, which may be useful in resolving and confirming species relationships if required (Zahniser and Dietrich 2010). Finally, as a result of the limited amount of molecular data for leafhoppers, new sequence information will be a valuable resource to entomologists.

A clear understanding of the mechanisms by which SbBMV persists on soybean will allow for appropriate control measures to be implemented. These may simply include good farming practices aimed at limiting the number of weeds in close proximity to soybean, or the use of systemic insecticides on perennial hosts and soybean. Future work will focus on investigating additional vectors of SbBMV, as well as the exact mechanisms of transmission

of SbBMV by these vectors. DNA sequences of additional accessions and species will also be determined to ensure that vector species can be identified correctly.

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## Tables

**Table 4.1. Nucleotide sequences (5'-3') of oligonucleotide primers used in this study.**

| Primer              | Sequence                                           |
|---------------------|----------------------------------------------------|
| Hex AF              | ATGGCTCGTACCAAGCAGACGGC                            |
| Hex AR              | ATATCCTTGGGCATGATGGTGAC                            |
| LCO1490             | GGTCAACAAATCATAAAGATATTGG                          |
| HC02198             | TAAACTTCAGGGTGACCAAAAAATCA                         |
| Soyblotch F         | CTTTGCCCAACTGGACTCCC                               |
| Soyblotch R         | TCCAAACAGTCTTCCCAGGC                               |
| Soyblotch R-Tag     | <u>GGCCGTCATGGTGGCGAATAAT</u> TCCAAACAGTCTTCCCAGGC |
| Tag-specific primer | AATAAATCATAAGGCCGTCATGGTGGCGAATAA                  |

The non-viral tag sequence on the Soyblotch R-Tag primer is underlined.

**Table 4.2. Leafhopper species identified during a 12 month survey in Brits, North West, South Africa.**

| Sub-family             | Genus                | Species               | Author/Note                                |
|------------------------|----------------------|-----------------------|--------------------------------------------|
| <i>Megophthalminae</i> | <i>Peragallia</i>    | <i>caboverdensis</i>  | Lindberg                                   |
|                        | <i>Igerna</i>        | <i>robustipenis</i>   | sp.n. Viraktamath & Linnavuori unpublished |
|                        | <i>Austroagallia</i> | <i>cuneate</i>        | Cogan                                      |
|                        | <i>Austroagallia</i> | <i>nighti</i>         | sp.n. Viraktamath & Linnavuori unpublished |
| <i>Typhlocybinae</i>   | <i>Epignoma</i>      | <i>natalensis</i>     | Dworakowska                                |
|                        | <i>Gambialoa</i>     | <i>newbyi</i>         | Ghuri                                      |
| <i>Deltocephalinae</i> | <i>Orosius</i>       | <i>albicinctus</i>    | Distant                                    |
|                        | <i>Cicadulina</i>    | <i>anestae</i>        | Van Rensburg                               |
|                        | <i>Cicadulina</i>    | <i>mbila</i>          | Naudé                                      |
|                        | <i>Nesoclutha</i>    | <i>erythrocephala</i> | Ferrari                                    |

**Table 4.3. Geographical origin, date of collection and accession information of *Peragallia caboverdensis* individuals used in this study.**

| <b>Accession number</b> | <b>Genbank accession number (<i>mtCOI/H3</i>)</b> | <b>Sex (M/F)</b> | <b>Geographical origin</b>      | <b>Collection date</b> |
|-------------------------|---------------------------------------------------|------------------|---------------------------------|------------------------|
| 18/6243                 | MH682003/MH682028                                 | M                | Brits, North West, South Africa | November 2017          |
| 18/6244                 | MH682005/MH682029                                 | M                | Brits, North West, South Africa | November 2017          |
| 18/6246                 | MH682017/MH682040                                 | M                | Brits, North West, South Africa | November 2017          |
| 18/6247                 | MH682006/MH682036                                 | M                | Brits, North West, South Africa | November 2017          |
| 18/6248                 | MH682007/MH682051                                 | M                | Brits, North West, South Africa | November 2017          |
| 18/6251                 | MH682018/MH682042                                 | M                | Brits, North West, South Africa | November 2017          |
| 18/6253                 | MH682024/MH682041                                 | M                | Brits, North West, South Africa | November 2017          |
| 18/6259                 | MH682026/MH682039                                 | M                | Brits, North West, South Africa | November 2017          |
| 18/6263                 | MH682008/MH682048                                 | M                | Brits, North West, South Africa | November 2017          |
| 18/6266                 | MH682027/MH682052                                 | M                | Brits, North West, South Africa | November 2017          |
| 18/6326                 | MH682015/MH682045                                 | M                | Brits, North West, South Africa | January 2018           |
| 18/6342                 | MH682020/MK682046                                 | F                | Brits, North West, South Africa | February 2018          |
| 18/6386                 | MH682023/MH682037                                 | F                | Brits, North West, South Africa | March 2018             |
| 18/6434                 | MH682019/MH682034                                 | M                | Brits, North West, South Africa | March 2018             |
| 18/6436                 | MH682009/MH682050                                 | M                | Brits, North West, South Africa | March 2018             |
| 18/6446                 | MH682016/MH682047                                 | M                | Brits, North West, South Africa | March 2018             |
| 18/6463                 | MH682010/MH682030                                 | M                | Brits, North West, South Africa | March 2018             |
| 18/6468                 | MH682011/MH682043                                 | M                | Brits, North West, South Africa | March 2018             |
| 18/6470                 | MH682021/MH682032                                 | M                | Brits, North West, South Africa | March 2018             |
| 18/6475                 | MH682012/MH682044                                 | M                | Brits, North West, South Africa | March 2018             |
| 18/6493                 | MH682022/MH682035                                 | M                | Brits, North West, South Africa | March 2018             |
| 18/6552                 | MH682013/MH682038                                 | M                | Brits, North West, South Africa | April 2018             |
| 18/6584                 | MH682014/MH682049                                 | M                | Brits, North West, South Africa | April 2018             |
| 18/6587*                | MH682025/MH682033                                 | M                | Brits, North West, South Africa | November 2017          |
| 18/6588*                | MH682004/MH682031                                 | F                | Brits, North West, South Africa | November 2017          |

\*Voucher specimens not subjected to Soyblotch RT-PCR and Soyblotch pss-RT-PCR analysis.

## Figures



**Figure 4.1.** Photograph of the latero-frontal view of *Peragallia caboverdensis* illustrating the characteristic round spots present on the pronotum and crown. Male specimen in voucher series under accession CCDL27772. Image taken with Zeiss Axio Zoom and Axiocam MRc camera.

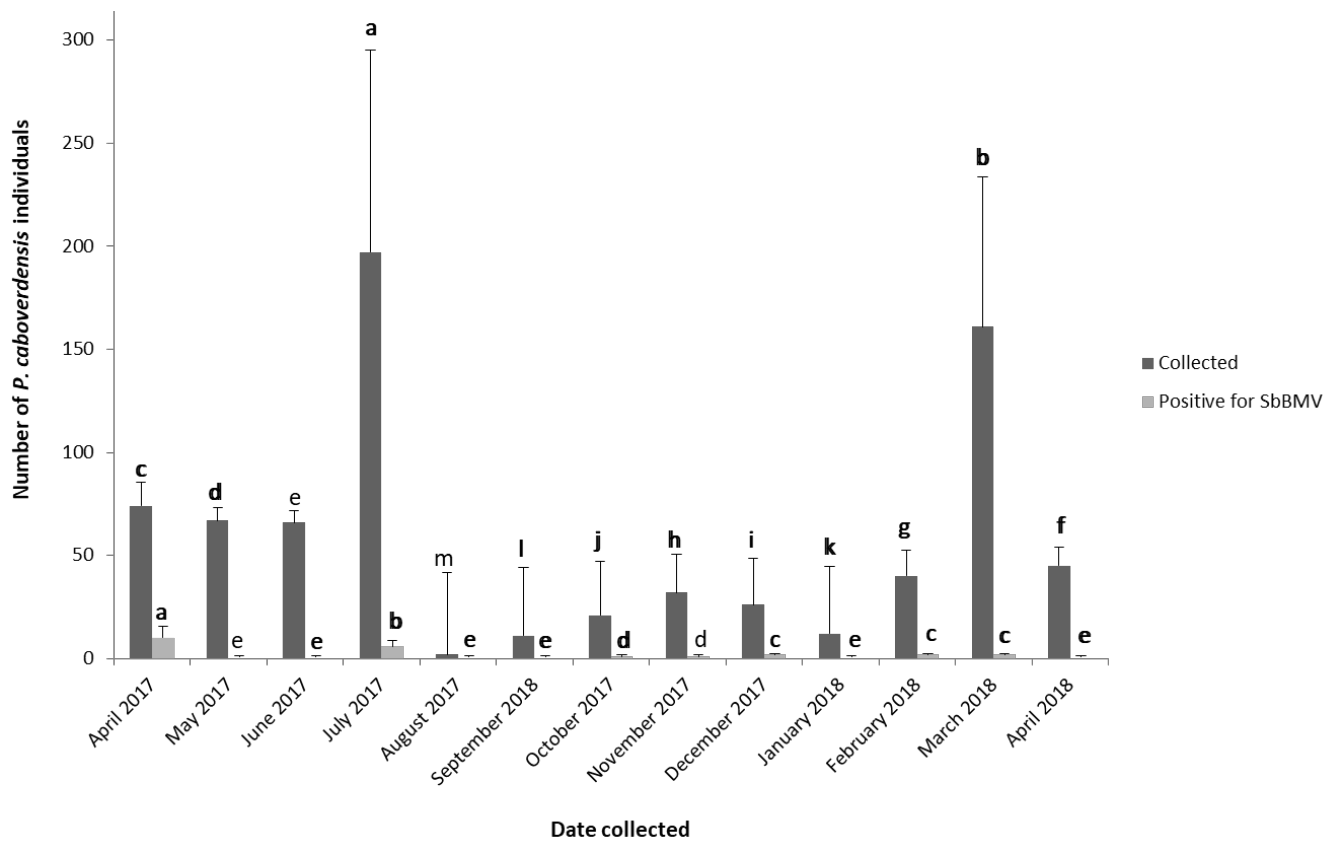


**Figure 4.2.** Number of *Peragallia caboverdensis* individuals collected and tested for SbBMV during a 13-month survey in Brits, North West, South Africa. Error bars represent standard deviation from the population mean. Statistical analysis was performed using analysis of variance (ANOVA) and a Tukey Honest Significant Differences test at a significance level of  $p < 0.05$  in R (R Core Team 2013). Bars displaying the same letters did not show significant differences at a confidence interval of  $p < 0.05$ .

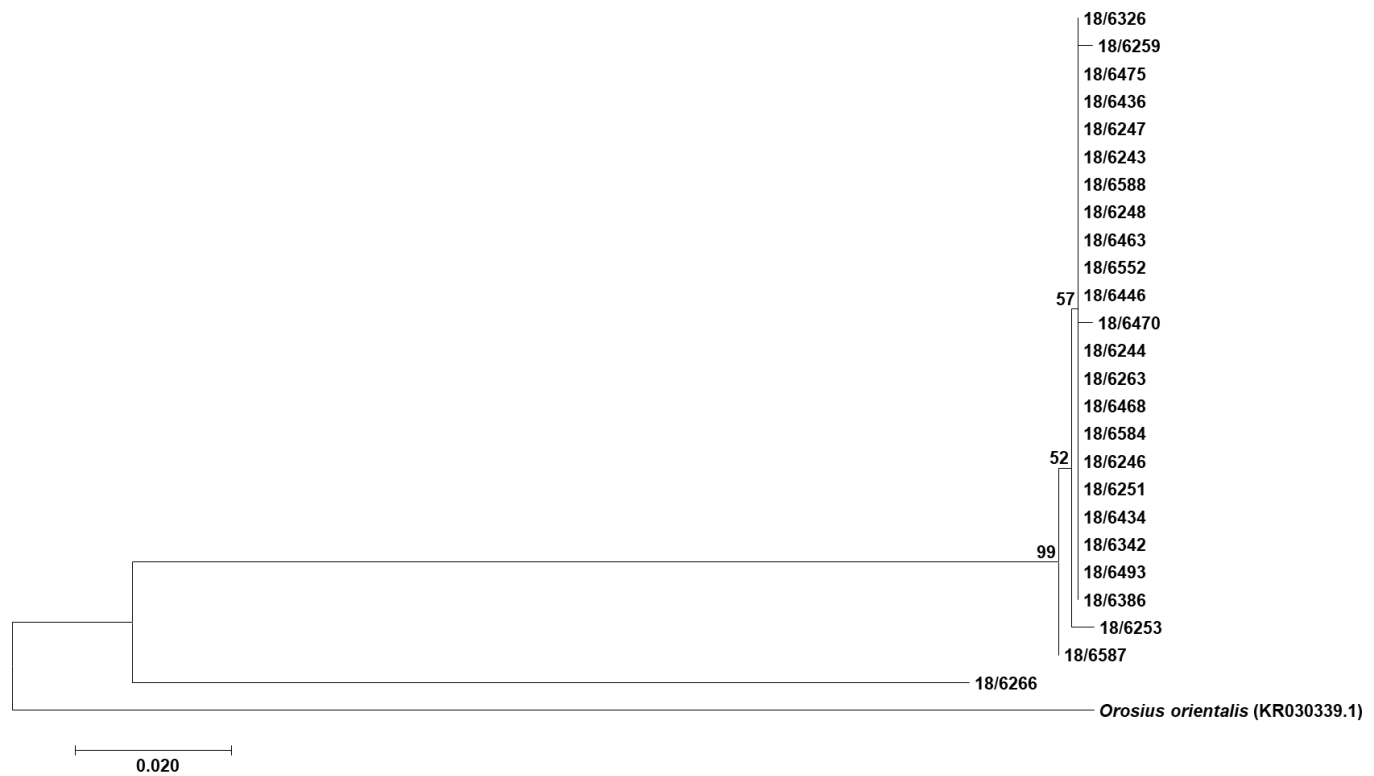


**Figure 4.3.** Neighbour-joining phylogenetic analysis of partial mitochondrial cytochrome oxidase subunit I (*mtCOI*) genes of 25 *Peragallia caboverdensis* individuals from different dates of collection in Brits, North West, South Africa. Phylogenetic tree is mid-point rooted, and bootstrap percentages (1000 replicates) are indicated at nodes of each clade. The common brown leafhopper *Orosius orientalis* (KR030339.1) was used as outgroup. Evolutionary analysis was conducted in MEGA 7.

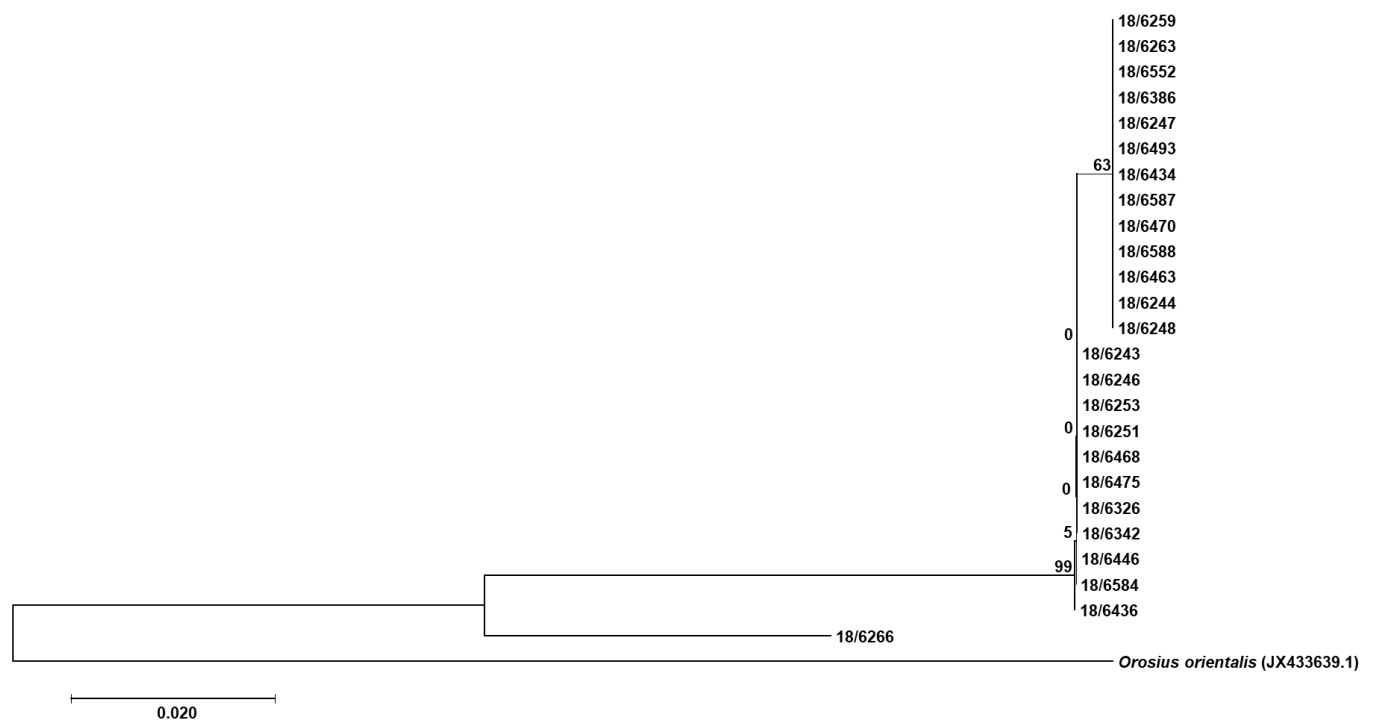


**Figure 4.4.** Neighbour-joining phylogenetic analysis of partial histone 3 (*H3*) genes of 25 *Peragallia caboverdensis* individuals from different dates of collection in Brits, North West, South Africa. Phylogenetic tree is mid-point rooted, and bootstrap percentages (1000 replicates) are indicated at nodes of each clade. The common brown leafhopper *Orosius orientalis* (JX433639.1) was used as outgroup. Evolutionary analysis was conducted in MEGA 7.

# Chapter 5

## **Diversity of partial RNA-dependent RNA polymerase gene sequences of Soybean blotchy mosaic virus isolates from different host-, geographical- and temporal origins**

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## 5.1 Abstract

Infection of soybean by the plant cytorhabdovirus Soybean blotchy mosaic virus (SbBMV) results in significant yield losses in the temperate, lower-lying soybean production regions of South Africa. A 277 bp portion of the RNA-dependent RNA polymerase gene of 66 SbBMV isolates from different hosts, geographical locations in South Africa, and time of collection spanning 16 years were amplified by RT-PCR and sequenced to investigate the genetic diversity of isolates. Phylogenetic reconstruction revealed three main lineages, designated Groups A, B and C, with isolates grouping primarily according to geographic origin. Pairwise nucleotide identities ranged between 85.7% and 100% among all isolates, with isolates in Group A exhibiting the highest degree of sequence identity, and isolates of Groups A and B being more closely related to each other than to those in Group C. This is the first study investigating the genetic diversity of SbBMV.

**Keywords:** Soybean blotchy mosaic virus; RNA-dependent RNA polymerase gene, maximum-likelihood analysis; pairwise nucleotide similarity

Soybean blotchy mosaic virus (SbBMV) is provisionally classified as a member of the genus *Cytorhabdovirus* within the family *Rhabdoviridae* based on viral shape, size, distribution of viral particles in plant cells and limited sequence information (Lamprecht et al. 2010). Members of the *Rhabdoviridae* have single-stranded or bipartite RNA genomes of negative polarity between 12 kb and 14.5 kb in size (Jackson et al. 2005; Dietzgen et al. 2017; Amarasinghe et al. 2017). Rhabdovirus genomes encode six structural proteins, the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase (L). Eighteen genera are recognized within the family *Rhabdoviridae*, of which four, *Nucleorhabdovirus*, *Cytorhabdovirus*, *Dichorhavirus* and *Varicosavirus* infect plants (Amarasinghe et al. 2017). The plant cyto- and nucleorhabdoviruses also encode one to four accessory proteins hypothesized to be involved in movement from cell to cell and other unknown functions.

Infection of soybean by SbBMV is characterized by blotchy mosaic symptoms on leaves early in the soybean production season, with symptom severity and incidence declining with time. The disease has only been detected in the lower lying soybean production areas of South Africa such as Limpopo, Mpumalanga, North West and KwaZulu-Natal (Pietersen and Garnett 1990; Pietersen et al. 1998). The virus has been shown to be mechanically transmissible to soybean and *Nicotiana benthamiana*, and the leafhopper *Peragallia carboverdensis* (Cicadellidae, Agalliinae) was identified as an insect vector of SbBMV (Lamprecht et al. 2010), but the whole genome sequence of SbBMV still needs to be determined.

Despite the economic importance of diseases caused by plant rhabdoviruses, they remain relatively poorly studied when compared to their animal-infecting counterparts. The amount of sequence information (both partial gene sequences and whole genome sequences) generated for plant rhabdoviruses has increased in the last few years, but studies on the evolutionary relationships and variability of plant rhabdoviruses still largely rely on portions of a gene sequence or full gene sequences of a few gene regions (Klerks et al. 2004; Revill et al. 2005; Talbi et al. 2011; Callaghan and Dietzgen 2005). The RNA-dependent RNA polymerase protein has conserved RNA-binding and polymerase domains, which are valuable for phylogenetic analysis, and consequently these are often used to infer evolutionary relationships and assess genetic diversity in plant rhabdoviruses (Bourhy et al. 2005; Klerks et al. 2004; Revill et al. 2005; Parrella and Greco 2016; Petrzik 2012).

The phylogenetic relationships and genetic diversity of SbBMV isolates have not been evaluated, and have important implications for viral diagnostics and the effective detection of all variants in a population, as well as understanding processes such as evolution and interactions between viruses and their hosts and vectors (Pappi et al. 2016). In this paper, partial sequences of the *L* gene of SbBMV isolates were generated by RT-PCR and Sanger sequencing and analyzed to assess the genetic diversity of SbBMV isolates from different plant and insect hosts, geographical origins in South Africa and three general collection

periods spanning 16 years. Maximum-likelihood (ML) phylogenetic reconstruction was used to infer phylogenetic relationships between isolates, and *p*-distances were calculated to assess genetic variability.

Isolates originating from plant hosts were obtained either as dried leaf material from the Virus, Antiserum and Seroreagent (PVAS) collection at the Agricultural Research Council-Plant Protection Research Institute (ARC-PPRI), in Pretoria, South Africa, or fresh leaf material displaying the typical blotchy mosaic symptoms associated with SbBMV. Samples were collected in the Brits (North West), Thabazimbi (Limpopo), Pretoria (Gauteng), Loskop Irrigation Scheme (Mpumalanga), Schoemanskloof (Mpumalanga), Dundee (KwaZulu-Natal) and Lusikisiki (Eastern Cape) areas of South Africa. Isolates obtained from asymptomatic alternative hosts of SbBMV, *Gymnosporia buxifolia* and *Lamium amplexicaule* were also included. Each plant was assigned a unique accession number and fresh plant material samples were stored at 4°C prior to homogenization with liquid nitrogen. Plant total RNA was extracted using 1.8 ml of CTAB buffer per sample according to the method described by White and co-workers (White et al. 2008) modified by the omission of spermidine in the CTAB buffer, and centrifugation for 15 min. Individuals of *P. caboverdensis* were collected from soybean cultivar trials in Brits, North West in which high incidences of the disease was observed, and preserved in 100% ethanol until processed. RNA was extracted from individuals of *P. caboverdensis* using the method described by Mallory and co-workers (Mallory et al. 2001) prior to RT-PCR and sequencing.

Partial *L* gene sequences of 66 isolates (Table 5.1) were determined. The Soyblotch F (5' CTTTGCCCAACTGGACTCCC 3') and Soyblotch R (5' TCCAAACAGTCTTCCCAGGC 3') primer pair were designed to amplify a 354 bp portion of 522 nt of the SbBMV *L* gene which is available in the National Centre for Biotechnology Information (NCBI) database (EU877231) (Lamprecht et al. 2010; Strydom and Pietersen 2018). RT-PCR using the Soyblotch primer pair was performed as previously described (Strydom and Pietersen 2018). Samples were submitted for Sanger sequencing using an ABI 3500xL automated sequencer

at the University of Pretoria, South Africa, and sequences subjected to a Basic Local Alignment Search Tool (BLASTn) search in the NCBI database in order to confirm the identity of isolates as SbBMV. Forward and reverse raw sequence reads were assembled using BioEdit software (Hall 1999), and consensus sequences were manually curated and aligned online using Mafft (Kato et al. 2002) with default parameters. Alignments were trimmed from both the 5' and 3' ends in BioEdit to minimise the influence of gaps present in alignments. Phylogenetic trees were constructed using the ML method based on the Tamura-Nei model (Tamura and Nei 1993) in MEGA software version 6 (Tamura et al. 2011) with 1000 replicates of bootstrap analysis, and Northern cereal mosaic virus (NCMV) (NC\_002251.1) as outgroup. Pairwise nucleotide distances (*p*-distance) for the same dataset was determined using SDT v.1.2 software (Muhire et al. 2013), and is presented as colour-coded blocks.

Analysis of 277 bp of the *L* gene of 66 SbBMV isolates using the ML method is presented in Figure 5.1. Phylogenetic analysis showed segregation of isolates into three main lineages, referred to here as groups A, B and C. Group A was dominated by isolates collected in the 1990's from the Loskop Irrigation Scheme, but also contains isolates from two other geographical locations (Schoemanskloof and Dundee) and the more recently isolated accessions 15/3086 (Loskop Irrigation Scheme), 15/3137 (Schoemanskloof), 16/4131 (Brits) and 17/5000 (Brits). Clade B consists of two sub-clades mostly of isolates collected from Lusikisiki in 2003 and Schoemanskloof during 2015, with the exception of isolates 15/3067 and 95/0015, which were collected in the Loskop Irrigation Scheme. Group C is mostly composed of isolates collected from soybean in Brits, but isolates from Thabazimbi (2), Pretoria (1) and Loskop Irrigation Scheme (3), the leafhopper vector *P. caboverdensis* and the alternative plant hosts *G. buxifolia* and *L. amplexicaule* represented the rest of the clade.

A clear correlation by grouping of isolates and geographic origin was observed. Group A, the two sub-clades within Group B and Group C were each dominated by isolates from a single geographic location, which were the Loskop Irrigation Scheme, KwaZulu-Natal and

Schoemanskloof, and Brits respectively. The presence of isolates from other locations within each of these clades indicated that these isolates were not geographically isolated with respect to gene flow, but rather that each region might select for specific strains or genotypes based on specific selection pressures present. As an example, the high variability and absence of isolates from Lusikisiki in Groups A and C and the limited presence of isolates from Schoemanskloof in the other groups might be the result of competitive exclusion. Enhanced interactions of isolates in Groups A and C with plant or insect hosts may lead to their apparent dominance, as also described in Lettuce necrotic yellows virus (LNYV) (Higgins et al. 2016). Soybean cultivar selection varies greatly in different regions, as does the natural vegetation, which may serve as alternative hosts, and these may act in strain selection, furthermore insect vectors often select for specific genotypes adapted to plant-vector systems (Ali et al. 2006). Multiple genetically distinct strains of SbBMV thus exist in each location, in which one genotype is favoured, increasing their prevalence.

Little is known of the epidemiology and variation of SbBMV. The association of Accession 15/3102 with mainly accessions from Brits suggests an introduction of SbBMV from the Loskop Irrigation Scheme into Brits in the early 1990's. The manner in which this may have occurred is unclear. Irrigation schemes are used at both Loskop and Brits, and similar crops are grown in both areas. Movement of plant material between these regions and/or enhanced vector breeding associated with high rainfall (irrigation) might have facilitated the introduction of *P. caboverdensis* into Brits. Lastly, rising temperatures can also increase the range, densities and migration potential of host plants and insect vectors (Canto et al. 2009). Lengthened breeding seasons for *P. caboverdensis* or expansion of the geographic ranges of alternative hosts, acting as a corridor for the spread of the vector, could also be plausible.

Clustering of isolates according to geographic origin has also been reported in other plant rhabdoviruses. Investigation of more than 1 kB of sequence data for each of 14 *N* and 19 *L* genes of Taro vein chlorosis virus isolates showed distinctive relationships between viral subgroups and geographical origin in the Pacific Islands (Revill et al. 2005). This was also

observed in a study which focussed on conserved regions of the *L* gene of 20 Eggplant mottled dwarf virus (EMDV) isolates, where phylogenetic analysis indicated two distinct genetic groups based on geographical origin (Parrella and Greco 2016). Furthermore, analysis of sequences corresponding to the *N*, *X*, *P*, *Y*, *G* and *L* genes, as well as the untranslated regions of EMDV from different alternative hosts generally clustered isolates into three subgroups based on geographical origin, and not host (Pappi et al. 2016).

The lack of a correlation between the clustering of isolates and host in EMDV isolates (Pappi et al. 2016) was also observed in this study. Accessions isolated from different hosts such as soybean, other alternative plant hosts and the insect vector *P. caboverdensis* all grouped together in clade C, illustrating an observed absence of divergence of SbBMV in different host backgrounds. A correlation by grouping of isolates and date of collection was also absent. Although the majority of isolates originating from the early 1990's clustered together in Group A, older collection accessions were also present in Groups B and C, and similarly, isolates collected more recently present in clade A. The isolates in the two sub-clades which represent Group B were also collected more than 10 years apart. Similarly, sequencing and phylogenetic analysis of the complete *N* gene of eight LNYV isolates from Australia also showed no temporal separation in subgroups (Callaghan and Dietzgen 2005). However, this is in contrast to later studies of LNYV which reported the clustering of more recently collected isolates in a subgroup, with older isolates forming a distinct clade (Higgins et al. 2016).

Pairwise nucleotide identities ranged between 98.5%-100%, 95.1%-100% and 92.7%-100% between SbBMV isolates in Groups A, B and C respectively (Figure 5.2). Between isolates of Groups A and B, the highest nucleotide similarity was 92.7%, and the lowest 90%. The minimum nucleotide identity between clade A and clade C was 86.3, and the maximum 92.1. The minimum sequence identity between clades B and C was 85.7%, and the maximum 92.4%. Isolates within Group A showed the least amount of genetic diversity, while those in Group C showed the highest amount of sequence diversity. Clades A and B appear to more

closely related to each other than to Group C, as indicated by the larger minimum and maximum nucleotide identities. It will be important to determine the whole genome of representative samples from each clade.

A previous study reported that NCMV had the highest nucleotide similarity to SbBMV (60.7%) among the cytorhabdoviruses (Lamprecht et al. 2010), and in this study, nucleotide similarities between SbBMV isolates and NCMV varied between 57.8% and 62.8 %. Despite the use of the closest member in the genus *Cytorhabdovirus* as outgroup, a long root was still obtained, which is attributed to the diversity present in different species of the cyto- and nucleorhabdoviruses. Cyto- and nucleorhabdoviruses show higher levels of genetic diversity than other genera in the *Rhabdoviridae* such as the lyssaviruses (Bourhy et al. 2005), which could be the result of purifying selection in the lyssaviruses or a more recent evolution.

In this study, we report an increase in the known distribution of SbBMV, now confirmed to also be present in the Eastern Cape, furthermore we demonstrate the presence of diverse populations of SbBMV isolates for the first time. In future, additional, longer gene regions or whole genome sequences should be used to confirm evolutionary relationships inferred here, and can be used to determine whether the genetic variation observed in a portion of the *L* gene also translates to the full genome sequence.

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## Tables

**Table 5.1. Host, geographical origin, year of collection and accession information of SbBMV isolates used in this study.**

| Isolate | Genbank<br>Accession<br>number | Host                            | Origin                                             | Year | Group |
|---------|--------------------------------|---------------------------------|----------------------------------------------------|------|-------|
| 91/0084 | MF964900                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1991 | A     |
| 91/0085 | MF964901                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1991 | A     |
| 91/0088 | MF964902                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1991 | A     |
| 91/0090 | MF964903                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1991 | A     |
| 91/0091 | MF964871                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1991 | A     |
| 91/0095 | MF964872                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1991 | A     |
| 92/0010 | MF964904                       | <i>Glycine max</i>              | Dundee, KwaZulu-Natal                              | 1992 | A     |
| 92/0026 | MF964905                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1992 | A     |
| 92/0030 | MF964907                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1992 | A     |
| 92/0033 | MF964908                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1992 | A     |
| 92/0034 | MF964906                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1992 | C     |
| 92/0404 | MF964909                       | <i>Glycine max</i>              | Thabazimbi, Limpopo, South Africa                  | 1992 | C     |
| 94/0398 | MF964873                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 1994 | A     |
| 94/2068 | MF964912                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1994 | A     |
| 94/2074 | MF964911                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1994 | A     |
| 94/2089 | MF964870                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1994 | A     |
| 95/0011 | MF964913                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1995 | A     |
| 95/0013 | MF964914                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1995 | A     |
| 95/0014 | MF964915                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1995 | A     |
| 95/0015 | MF964916                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1995 | B     |
| 95/0017 | MF964917                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 1995 | A     |
| 95/0038 | MF964918                       | <i>Glycine max</i>              | Thabazimbi, Limpopo, South Africa                  | 1995 | C     |
| 95/0073 | MF964919                       | <i>Glycine max</i>              | Pretoria, Gauteng, South Africa                    | 1995 | C     |
| 03/4013 | MF964910                       | <i>Glycine max</i>              | Lusikisiki, Eastern Cape, South Africa             | 2003 | B     |
| 03/4025 | MF964896                       | <i>Glycine max</i>              | Lusikisiki, Eastern Cape, South Africa             | 2003 | B     |
| 03/4029 | MF964897                       | <i>Glycine max</i>              | Lusikisiki, Eastern Cape, South Africa             | 2003 | B     |
| 03/4030 | MF964898                       | <i>Glycine max</i>              | Lusikisiki, Eastern Cape, South Africa             | 2003 | B     |
| 03/4033 | MF964899                       | <i>Glycine max</i>              | Lusikisiki, Eastern Cape, South Africa             | 2003 | B     |
| 14/3001 | MF964874                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2014 | C     |
| 14/3002 | MF964875                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2014 | C     |
| 14/3005 | MF964876                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2014 | C     |
| 15/3002 | MF964877                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2015 | C     |
| 15/3006 | MF964878                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2015 | C     |
| 15/3011 | MF964879                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2015 | C     |
| 15/3067 | MF964880                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 2015 | B     |
| 15/3069 | MF964881                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 2015 | C     |
| 15/3086 | MF964882                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 2015 | A     |
| 15/3102 | MF964883                       | <i>Glycine max</i>              | Loskop Irrigation Scheme, Mpumalanga, South Africa | 2015 | C     |
| 15/3137 | MF964884                       | <i>Glycine max</i>              | Schoemanskloof, Mpumalanga, South Africa           | 2015 | A     |
| 15/3142 | MF964885                       | <i>Glycine max</i>              | Schoemanskloof, Mpumalanga, South Africa           | 2015 | B     |
| 15/3146 | MF964886                       | <i>Glycine max</i>              | Schoemanskloof, Mpumalanga, South Africa           | 2015 | B     |
| 15/3147 | MF964887                       | <i>Glycine max</i>              | Schoemanskloof, Mpumalanga, South Africa           | 2015 | B     |
| 15/3155 | MF964888                       | <i>Glycine max</i>              | Schoemanskloof, Mpumalanga, South Africa           | 2015 | B     |
| 15/3156 | MF964889                       | <i>Glycine max</i>              | Schoemanskloof, Mpumalanga, South Africa           | 2015 | B     |
| 15/3250 | MF964890                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2015 | C     |
| 16/4129 | MF964893                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2016 | C     |
| 16/4131 | MF964894                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2016 | A     |
| 16/4132 | MF964920                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2016 | C     |
| 16/4134 | MF964921                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2016 | C     |
| 16/4427 | MF964892                       | <i>Gymnosporia buxifolia</i>    | Brits, North West, South Africa                    | 2016 | C     |
| 16/4712 | MF964891                       | <i>Lamium amplexicaule</i>      | Brits, North West, South Africa                    | 2016 | C     |
| 17/5000 | MF964922                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2017 | A     |
| 17/5002 | MF964923                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2017 | C     |
| 17/5003 | MF964924                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2017 | C     |
| 17/5004 | MF964925                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2017 | C     |
| 17/5005 | MF964926                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2017 | C     |
| 17/5006 | MF964927                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2017 | C     |
| 17/5008 | MF964928                       | <i>Glycine max</i>              | Brits, North West, South Africa                    | 2017 | C     |
| 17/5501 | MF964929                       | <i>Peragallia caboverdensis</i> | Brits, North West, South Africa                    | 2017 | C     |
| 17/5503 | MF964930                       | <i>Peragallia caboverdensis</i> | Brits, North West, South Africa                    | 2017 | C     |
| 17/5505 | MF964931                       | <i>Peragallia caboverdensis</i> | Brits, North West, South Africa                    | 2017 | C     |
| 17/5506 | MF964932                       | <i>Peragallia caboverdensis</i> | Brits, North West, South Africa                    | 2017 | C     |
| 17/5507 | MF964933                       | <i>Peragallia caboverdensis</i> | Brits, North West, South Africa                    | 2017 | C     |
| 17/5508 | MF964934                       | <i>Peragallia caboverdensis</i> | Brits, North West, South Africa                    | 2017 | C     |
| 17/5539 | MF964935                       | <i>Peragallia caboverdensis</i> | Brits, North West, South Africa                    | 2017 | C     |
| 17/5633 | MF964895                       | <i>Peragallia caboverdensis</i> | Brits, North West, South Africa                    | 2017 | C     |

## Figures

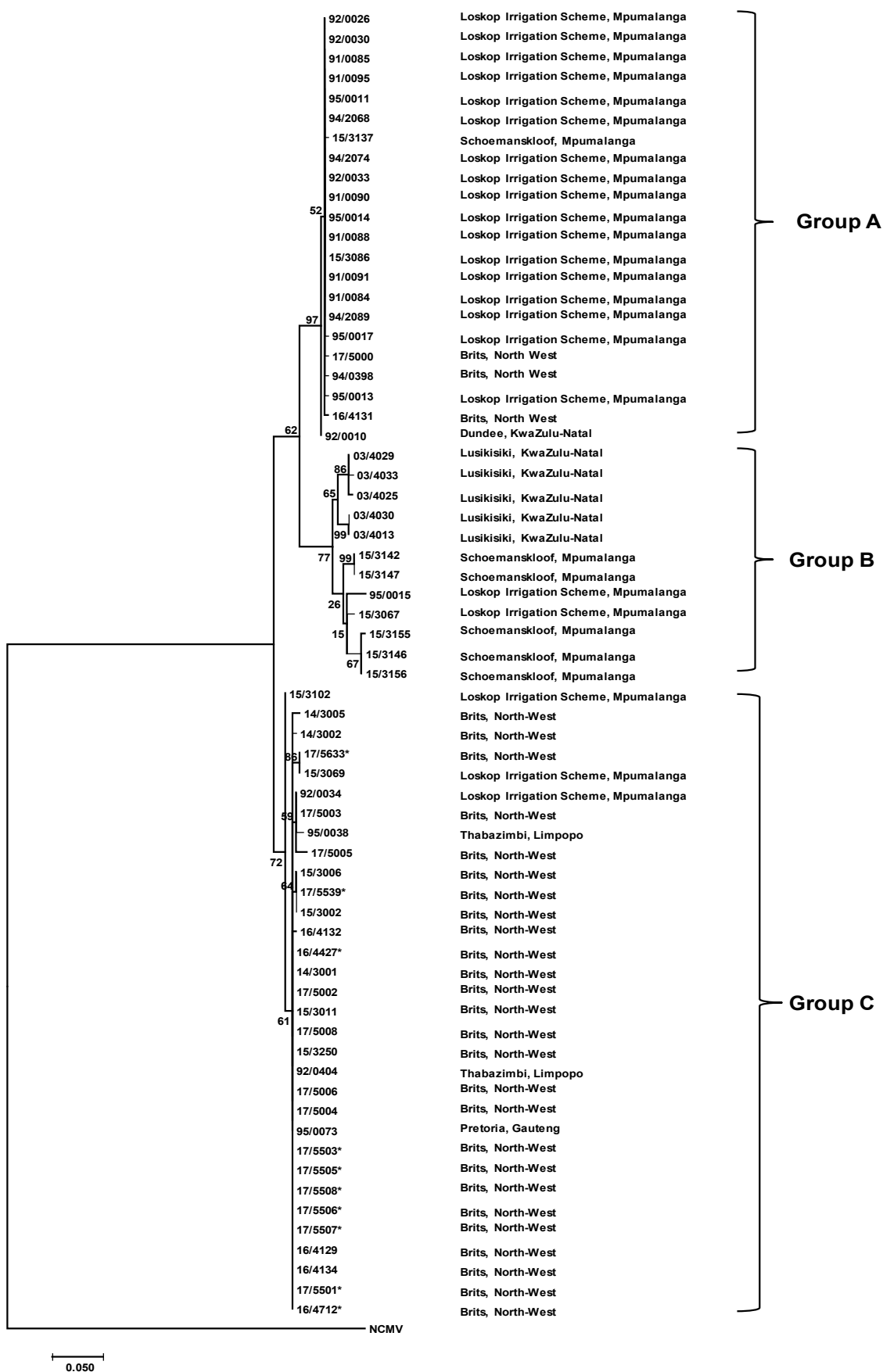


**Figure 5.1.** Maximum-likelihood phylogenetic analysis of partial RNA-dependent RNA polymerase (L) genes of 66 Soybean blotchy mosaic virus isolates from different geographical origins and hosts. Phylogenetic tree is mid-point rooted, and bootstrap percentages (1000 replicates) are indicated at nodes of each clade. Northern cereal mosaic virus (NC\_002251.1) was used as outgroup. Evolutionary analysis was conducted in MEGA 6.

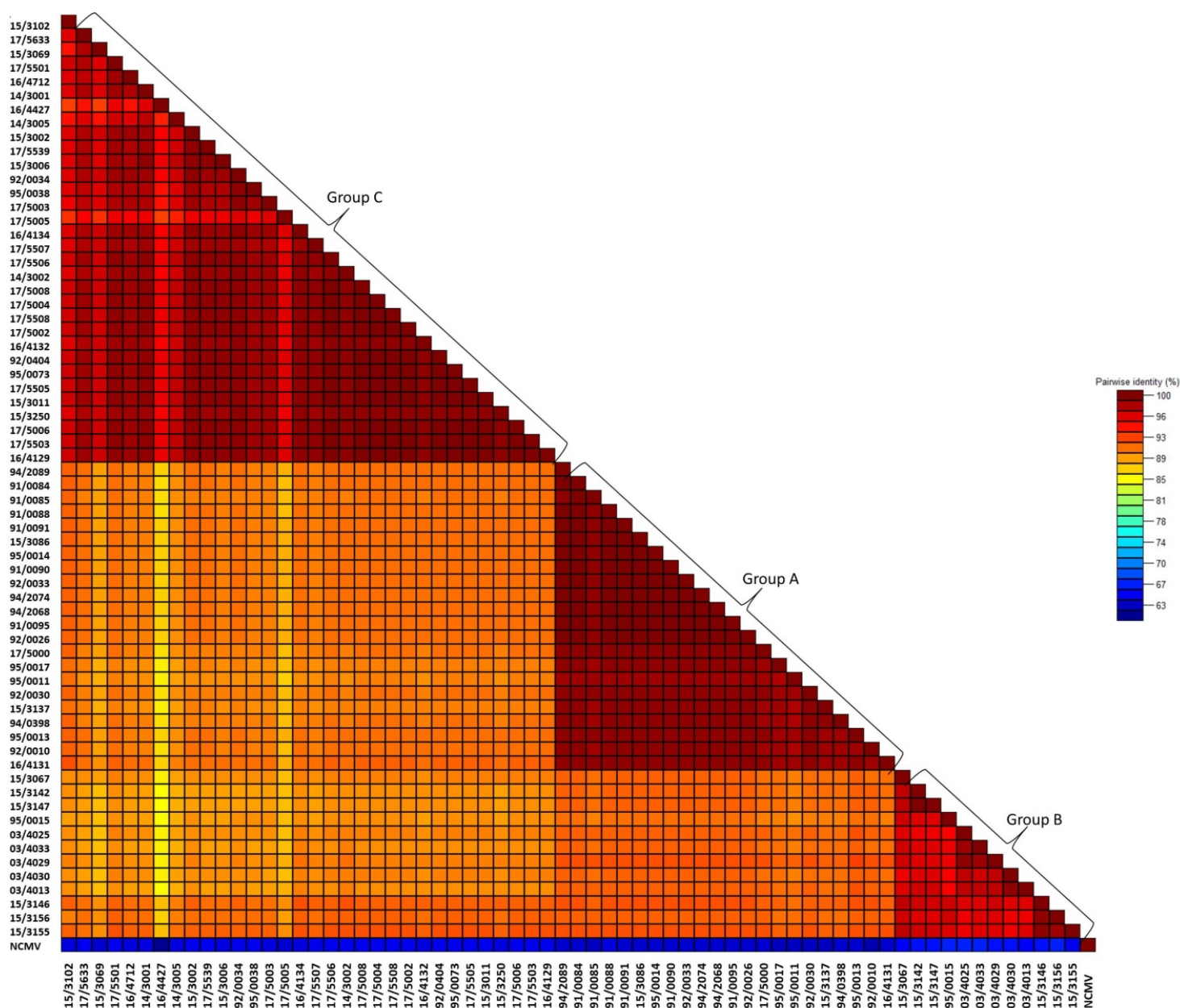


**Figure 5.2.** Graphical representation of pairwise nucleotide identities of 66 Soybean blotchy mosaic virus isolates used in this study with percentage identity scale. Pairwise nucleotide identities were obtained with SDT v1.2 software. Northern cereal mosaic virus (NC\_002251.1) was used as outgroup in phylogenetic analysis, and is included here. Origin and hosts of isolates are listed in Table 5.1.

# **Chapter 6**

## **General conclusion**

## 6.1 General discussion and future prospects

The global importance of soybean relies mainly on its use as animal feed, but it may become increasingly important as an alternative, more affordable source of protein for human consumption in future (Goldsmith 2008). Predicted changes in the availability of freshwater associated with climate change will significantly affect beef, poultry and pork production as a result of their large water footprint (Mekonnen and Hoekstra 2012), leading to soybean with its comparatively lower water footprint becoming a more sustainable form of protein. The viable production of soybean requires the limitation of yield losses as a result of biotic and abiotic factors. This thesis focused on characterizing Soybean blotchy mosaic virus (SbBMV), a plant rhabdovirus which causes significant yield losses in soybean in north and eastern South Africa.

The cytorhabdovirus SbBMV was first detected in South Africa in the early 1990's, and its leafhopper vector *Peragallia caboverdensis* (Cicadellidae: Megophthalminae: Agalliini) was also later identified (Pietersen 1993; Pietersen and Garnett 1990; Pietersen et al. 1998; Lamprecht et al. 2010). The blotchy mosaic symptoms on leaves that are associated with SbBMV infection of soybean is present early in each new soybean season. As a result of the annual nature of soybean production, this suggests that SbBMV reservoirs (plant or insect vector) or transmission through seed are possible mechanisms which facilitate the re-introduction of SbBMV onto soybean each year. The first aim of this project was to investigate whether alternative plants hosts of SbBMV could be identified, to determine the presence or absence of SbBMV transmission through soybean seed and to examine the role of *P. caboverdensis* in the persistence of SbBMV.

Three new plant hosts of SbBMV were identified by RT-PCR. The three species included one perennial, leguminous tree, *Gymnosporia buxifolia*, and two common weeds, *Lamium amplexicaule* and *Flaveria bidentis*. The perennial nature of *G. buxifolia* implicates the presence of a more permanent source of SbBMV for spread to soybean by *P.*

*caboverdensis*. The SbBMV-positive *L. amplexicaule* accession was collected in the soybean off-season (winter months), and as for *G. buxifolia*, thus also represents a SbBMV reservoir in the winter months from which SbBMV can be spread to soybean in the new production season. These plant species can only serve as inoculum sources for spread to soybean if *P. caboverdensis* is also present, thus the persistence of *P. caboverdensis* was also investigated during this study.

Transmission of SbBMV through the seed of four soybean cultivars grown commercially in the North West province of South Africa was not detected in 2, 829 seedlings. Transmission through seed has not been reported for plant rhabdoviruses to date (Jackson et al. 2005; Dietzgen et al. 2017). The possibility that SbBMV might also be transmissible in seed of an alternative host, which can facilitate the re-emergence and spread of SbBMV each year cannot be ignored however. The lack of demonstrated seed transmission implies that transmission of SbBMV by *P. caboverdensis* or other leafhopper vectors is probably an important method of spread of SbBMV, as mechanical transmission was previously reported to occur at low frequencies (Lamprecht et al. 2010).

The role of *P. caboverdensis* in the persistence of SbBMV on soybean was investigated with a survey aimed at determining the prevalence of *P. caboverdensis* throughout the year and efforts to determine the exact mode of transmission of SbBMV by *P. caboverdensis*. The survey revealed a year-round presence of SbBMV in the area investigated, and SbBMV-positive accessions were detected in both the summer and winter months, which roughly corresponds to the soybean production and off-seasons. This permanent presence of *P. caboverdensis* confirmed its potential to survive on other plant hosts in the absence of soybean. This would be a prerequisite for the spread of SbBMV to soybean from reservoir plant hosts. The co-occurrence of *P. caboverdensis* and SbBMV-positive alternative hosts in both the summer and winter months represents a potential mechanism whereby SbBMV persists between soybean seasons.

In order to establish whether propagation of SbBMV in *P. caboverdensis* contributes to the seasonal persistence of SbBMV, a positive strand-specific RT-PCR (pss-RT-PCR) was developed to specifically detect the positive sense RNA strand of SbBMV. In a virus with a genomic RNA of negative polarity such as SbBMV this indicates replication, as positive sense RNAs act as replicative intermediates during the replication process. None of the *P. caboverdensis* accessions which tested positive for the presence of SbBMV using the standard diagnostic RT-PCR tested positive for replication of SbBMV using the pss-RT-PCR. Transmission of SbBMV in a persistent, propagative manner by *P. caboverdensis* was thus not detected in this study.

This may be attributed to limitations associated with the design of the study, the presence of additional vectors or transmission of SbBMV in a non-propagative manner. However, this is unlikely as all plant rhabdoviruses for which mode of transmission by their insect vectors have been studied were transmitted in a persistent, propagative manner (Barandoc-Alviar et al. 2016; Whitfield et al. 2015; Ammar and Hogenhout 2008; Conti 1985; Ammar and Nault 1985; Falk and Tsai 1985; Todd et al. 2010). This may suggest the presence of another leafhopper vector. The establishment of a *P. caboverdensis* colony will be an important tool to conclusively determine the mode of SbBMV transmission by *P. caboverdensis*, by monitoring viral load and replication over time using RT-qPCR and the Soyblotch pss-RT-PCR respectively.

The first DNA sequence information for *P. caboverdensis* was also generated in this study. Partial sequences of the mitochondrial cytochrome c oxidase subunit I (*mtCOI*) and histone 3 (*H3*) gene regions was determined, and this will greatly aid identification of *P. caboverdensis* of all life stages, especially when morphological expertise is not available. Sequence information will also be a useful character for taxonomists tasked with reviewing the classification of the leafhoppers. For the number and geographic origin of *P. caboverdensis* accessions used in this study, these two gene regions appear to exhibit sufficient amounts of intraspecific sequence conservation for accurate species identification.

Nevertheless, in future, accessions from other geographical locations and closely related species should also be included to confirm amounts of intraspecific variation among geographic locations and the necessary amount of interspecific variation for species delineation using these markers.

The second aim of this study was to assess the genetic diversity present in populations of SbBMV. Isolates from seven different locations, insect, soybean and alternate plant hosts and a collection period spanning 16 years were included in the study. The 66 SbBMV accessions grouped into three main lineages, each of which was mostly dominated by isolates from a specific location. The dominance of specific genotypes was attributed to area-specific selection pressures and not an absence of gene flow, as all three the main lineages also included isolates originating in other locations (Higgins et al. 2016; Ali et al. 2006). The grouping of SbBMV isolates according to geographic origin and not host or date of collection has also been observed in other nucleo- and cytorhabdoviruses.

Future studies should expand on this aspect by including additional and longer gene regions. The determination of the whole genome sequence of SbBMV will also allow for a phylogenomics approach, in which results obtained in this study can be compared to the levels of diversity present across the whole genome. The diagnostic PCR developed in this study appears to pick up all variants of SbBMV, as illustrated by its use in the diverse populations identified in this study.

The findings of this study allows for recommendations on control strategies aimed at limiting the production losses associated with SbBMV infection. A two-tiered approach of eliminating both the sources of inoculum and the agent of spread should be followed. Known alternate host weeds, trees and volunteer plants should be treated with systemic insecticides. Secondly, the application of systemic insecticides in soybeans themselves will aid the control of leafhopper populations, limiting the potential of virus spread.

In the long term, the identification of resistance (R) genes in soybean for incorporation into susceptible cultivars, whether by a cisgenic or transgenic approach, or the deployment of pathogen-derived genes can also be considered. The quasispecies nature of plant RNA virus populations complicates the control of plant virus diseases in crops as it allows for emergence, virulence and host switching (Martinez et al. 2011). The large reservoir of viral variants facilitates rapid adaptation to the selective pressures exerted by the deployment of plant R genes or pathogen-derived resistance through either conventional breeding or transgenic approaches in crops. Coupled with the threat of expanding viral host ranges as a result of changes in the host range of insect vectors in response to global warming, changing agricultural practices and the increasingly more widespread movement of plant material, the control of plant viral disease will become more challenging in future (Roossinck 1997).

Despite the economic importance of plant rhabdoviruses globally, this group of viruses have not been subject to extensive studies. Characterization of a new member of this group adds to the existing body of knowledge on the epidemiology, spread and diversity of this interesting family of viruses.

## 6.2 Literature cited

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