WEEDS: RESERVOIRS OF VECTOR-BORNE VIRAL PATHOGENS IN SOUTH AFRICA

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FRONTISPIECE

Virus-like symptoms observed on Solanum nigrum (left) and Datura stramonium (right) alongside tomato (*Solanum lycopersicum* L.) crop fields in South Africa

DISSERTATION ABSTRACT

Weeds have the potential to alter the function of ecosystems either as pests themselves or by harbouring vectors and vector-borne diseases. Their role as reservoirs of viral pathogens has had a significant bearing on viral epidemiology in many parts of the world. However past scientific research has largely overlooked the impact of weeds on viral ecology. Only in recent years has virological research shifted to include the role of weeds in viral epidemiology and ecology. While there is an increasing number of reports on viruses identified on weeds globally, there is a significant lack of information on the distribution of weeds and the viruses they harbour in South Africa.

Therefore, a survey was conducted across major tomato growing areas located throughout various provinces in South Africa to identify weed species that harbour vectors and vector-borne viral diseases. Fifteen weeds species growing around tomato crop fields were found exhibiting virus-like symptoms in the presence of excessively high aphid, thrip and whitefly populations. Symptoms observed on weeds and tomato crops ranged from mosaics, mottling, necrotic spots, leaf curling, interveinal chlorosis and purple discoloration of leaves. *Tomato spotted wilt virus* (TSWV), *Potato virus Y*, *Tomato torrado virus* and *Tomato chlorosis virus* were positively identified using reverse transcription polymerase chain reaction (RT-PCR), and confirmed using BLAST, on *Amaranthus thunbergii*, *Physalis peruviana, Datura stramonium* and *Solanum nigrum* respectively. These viruses were also detected on tomato crops. These results confirm that weeds act as reservoirs of viral pathogens which may cause serious repercussions for crop production. The density and distribution of weeds in South Africa emphasise their role in viral epidemiology. Therefore stringent control measures should be employed to reduce the rate of vector transmitted viruses.

TSWV is ranked among the most destructive and complex viral pathogens globally. It has an extensive host range and global distribution, yet very few TSWV isolates have been completely sequenced. Most sequences of TSWV isolates have only been partially characterised. Therefore the aim of this study was to conduct a complete genome analysis of a South African TSWV isolate. Next-generation

sequencing technology was used to obtain the complete genome of TSWV. The complete genome (LK-1), as well as the genome organisation of TSWV, was obtained and subjected to phylogenetic and recombination analysis. Phylogenetic analysis showed geographically diverse phylogenetic relationships of each open reading frames of TSWV. One recombination event on the NSs ORF of S segment was detected. It seems that most recombination events are limited to this particular region.

In this study, the first complete genome of a South African TSWV isolate is reported. Additional full genome sequences of other South African TSWV isolates will enable a comprehensive population study of TSWV in South Africa. This information will contribute to the understanding of the emergence and evolution of TSWV and its adaptation to new hosts.

DECLARATION

I, **Lavinia Kisten**, declare that:

I. The research reported in this dissertation, except where otherwise indicated, is my original work.

II. This dissertation has not been submitted for any degree or examination at any other university.

III. This dissertation does not contain other persons' data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

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“But they that wait upon the LORD shall renew their strength; they shall mount up with wings as eagles; they shall run and not be weary; and they shall walk, and not faint.” – Isaiah 40:31.

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DEDICATION

To my mother for giving me the courage to reach for more than I thought I was capable of; for teaching the value of education and perseverance to me.

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DISSERTATION INTRODUCTION

Food security has become an alarming crisis in many parts of the world. Pests and diseases contribute significantly to the imbalance of food demand and agricultural output. Between 20 – 40 % of global food production is lost to plant diseases (Savary *et al.*, 2012). Viral diseases, in particular, are a major problem due to their insidious nature. They rank second only to fungal diseases in terms of economic damage caused (Otim-Napea *et al.*, 2003). Among the many emerging infectious diseases of plants, 47% are of viral aetiology (Fereres, 2015).

One crop that is particularly plagued by viral diseases is the cosmopolitan tomato (*Solanum lycopersicum* L.) crop. Tomato is one of the most widespread and important vegetable crops in South Africa, second only to the potato (*Solanum tuberosum* L.) crop (Anonymous, 2011a). It is a popular crop among both commercial and subsistence farmers and approximately 6000 hectares of arable land in South Africa is used for tomato production (Anonymous, 2011a). In 2010 the tomato industry was estimated to be worth R1.6 billion (Anonymous, 2011b).

The relationship between virus and host is considerably complex. Historically, much emphasis and research have been placed on the interaction between virus, crop and vector. The proverbial dark horse in this scenario that has been largely overlooked is weeds. Weeds were previously known to act as alternate hosts of plant viruses, however, their role in virus ecology and epidemiology was thought to be inconsequential (Mubin *et al.*, 2009). Recent studies show that weeds play a pivotal role in virus distribution and overwintering and as initial sources of inoculum (Asala *et al.*, 2014). Due to the lack of effective weed management strategies in agricultural settings, virus infections in crop fields are exacerbated. Even though virological research is now encompassing the study of weeds, much is still unknown about its impact on virus epidemiology.

PROJECT OBJECTIVES

Against this background, this research project was undertaken with the aim to identify weed species harbouring plant viruses in major tomato production areas in South Africa. The objectives of this study entailed:

1. A survey of viruses infecting weeds growing alongside tomato crops in South Africa.
2. Identification of weed plants.
3. Identification and characterization of viruses infecting weeds.
4. Whole genome analysis of a TSWV isolate using Next-generation sequencing.

ORGANIZATION OF DISSERTATION

This dissertation comprises of four chapters. Chapter one is a review of current literature with emphasis on the role of weeds in virus epidemiology. Chapter two addresses specific objectives 1-3. Chapter three focuses on specific objective 4. Chapter four provides an overview of the major findings of this study and suggests future research to fill information gaps identified. Chapters two and three were independent studies and were written in the form of distinct research chapters, each following the format of a stand-alone research paper. This format is the standard dissertation model that has been adopted by the University of KwaZulu-Natal as it facilitates the publishing of research out of the dissertation more efficiently than the older monograph form of dissertation. As such, there is some unavoidable repetition of references, methods and some introductory information between chapters.

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CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

This review focuses on the role of weeds in the epidemiology of important viruses infecting tomato crops. Background information pertaining to the classification of weeds, advances in weed management, their role in agriculture and virus epidemiology was gathered. Additional information on important vectors and vector-borne viral diseases was also reviewed.

Weeds are important biotic constraints of ecosystems, displaying a high degree of adaptability and colonising efficiency (Anonymous, 2014; Clements *et al.*, 2014). Weeds alter the function of ecosystems by decreasing biodiversity and promoting habitat loss of competing plant species (Kaur *et al.*, 2014). The distribution of weeds has been exacerbated by human influence (Rouget *et al.*, 2015). Agricultural productivity is threatened by weeds reducing available nutrients to the crop plants and acting as reservoirs of vector-borne viral diseases (Vafaei and Mahmoodi, 2015).

Viruses are intracellular entities that parasitize a host's molecular pathways to replicate itself and thereby spread the infection within the host (Heinlein, 2015). Currently, over a thousand plant viruses have been identified globally with the vast majority of these infecting crop plants (Roossinck, 2015). The top ranking viruses known to affect the tomato crops are *Tobacco mosaic virus* (TMV), *Tomato spotted wilt virus* (TSWV), *Tomato yellow leaf curl virus* (TYLCV), *Potato virus Y* (PVY), *Potato virus X* (PVX) and *Tomato bushy stunt virus* (TBSV) (Rybicki, 2015). Plant viruses have the potential to cause devastating crop damage resulting in significant yield losses.

The role of weeds as reservoirs of viral pathogens has significantly impacted viral epidemiology in many parts of the world including South Africa. They serve as potential initial sources of viral inoculum in crop fields (Prajapat *et al.*, 2014).

Furthermore, weeds are known to harbour viral vectors thereby contributing to the persistence of viral diseases in agroecosystems worldwide (Asala *et al.*, 2014).

1.2 WEED ECOLOGY

Weeds are a dynamic threat to the natural environment and agricultural productivity. They are highly adaptable pests with vast distribution patterns which have been exacerbated by human influence (Rouget *et al.*, 2015). Weed associations change gradually over time and are closely linked to agricultural practices (Clements *et al.*, 2014). In nature, no plant is considered a weed, however, ecological succession results in growing conditions being favourable for some plants thus allowing them to flourish and outcompete others and this effect is exacerbated by anthropogenic activities (Schonbeck, 2013).

1.2.1 Classification of weeds

Of the 250 000 known plant species, 3% are considered weed species (Lingenfelter and Hartwig, 2013). In addition to botanical classification, weeds can be grouped into seven major categories based on morphology, life cycle, habitat, origin, nature of stem, soil pH and special classification.

1.2.1.1 Morphology (Kwaga *et al.*, 2014)

Weed morphology is the most commonly used system of classification. Using this system of classification weeds are categorised into three groups:

- Grasses – are characterised by long and narrow leaves with hollow stems.
- Sedges – are marked by modified stems surrounded by whorls of leaves.
- Broadleaf weeds – are distinguished by expanded leaf blades. The majority of weed families fall under this category.

1.2.1.2 Lifecycle (Lingenfelter and Hartwig, 2013)

- Annuals – are weed species that survive for a season or a year and complete their lifecycle within that season or year.
- Biennials – weeds complete their life cycle within two seasons/years.

- Perennials – survive for more than two years. They are able to withstand adverse environmental conditions.

1.2.1.3 Habitat (Kwaga *et al.*, 2014)

- Terrestrial weeds – complete their lifecycle on dry land.
- Aquatic weeds – are adapted to grow in submerged soil conditions.
- Semi-aquatic – show some degree of tolerance to waterlogged soils.

1.2.1.4 Origin (Singh, 2008)

- Indigenous weeds – are native to a particular country
- Introduced or exotic weeds – are introduced from other countries. They pose major threats to the biodiversity of ecosystems and control is difficult.

1.2.1.5 Nature of stem (Singh, 2008)

- Woody and semi-woody – are collectively known as brush weeds
- Herbaceous – are distinguished by green succulent stems.

1.2.1.6 Soil pH (Singh, 2008)

- Acidophile – acid soil weeds
- Basophile – saline & alkaline soil weeds
- Neutrophile – weeds growing in neutral soils

1.2.1.7 Special Classification (Singh, 2008)

- Poisonous weeds – can cause ailments on animals and people and may result in death.
- Parasitic weeds – either totally or partially depend on a host plant for its metabolic functions.
- Aquatic weeds – grow in water and complete part of its life cycle in water. They are further divided into four groups: submersed, emersed, marginal and floating weeds.

1.2.2 Weeds in agriculture

Weeds are often inconspicuous threats in agriculture, which evolve in response to agricultural management practices. Annual economic losses caused by weeds

worldwide is estimated at US$ 400 billion (Clements *et al.*, 2014). Weeds are important components in cropping systems either by acting as a pest itself or by harbouring vectors and vector-borne diseases (Van Bogaert *et al.*, 2015). They inhibit crop growth by outcompeting crops for essential nutrients, space and water (Goyal *et al.*, 2015). Furthermore, weeds play a critical role in influencing viral disease incidence and spread resulting in significant yield losses (Prajapat *et al.*, 2014).

In addition to being agricultural pests, some weed species have noxious effects that are harmful to plant, animal and human health. Examples of these weeds are the notorious *Parthenium hysterophorus* L. (famine weed) and *Datura stramonium* L. (common thorn apple), both ranked as category 1b invasive plants according to the South African Alien and Invasive Species lists of the National Environmental Management: Biodiversity Act (NEMBA) of 2004 (Julyan, 2014; NEMBA, 2016). *P. hysterophorus* L., in particular, has garnered much attention globally and is considered one of the most destructive terrestrial weed species in the world (Fig. 1.1). It is an aggressive coloniser, threatening the biodiversity of ecosystems worldwide (Anonymous, 2014). Parthenium has an allelopathic effect on surrounding plants, impeding germination and growth of plants (Kaur *et al.*, 2014). In animals, it causes severe dermatitis and if ingested, mouth ulcers form and cattle develop bittermilk and tainted flesh (Adkins and Shabbir, 2014). In extreme cases, death may occur if a significant amount Parthenium is consumed (Kilewa and Rashid, 2014). Parthenium exposure to humans cause contact dermatitis and respiratory disorders (Kaur *et al.*, 2014).

Figure 1.1: Famine weed (*Parthenium hysterophorus* L.) (Anonymous, 2014)

Weeds have a controversial nature but despite their reputation, some weed species have beneficial attributes. One such weed is the edible pigweed (*Amaranthus* spp.) which is said to be one of Africa's most nutritious and widespread leafy vegetable (Achigan-Dako *et al.*, 2014). It is a valuable and popular food source in informal African markets (Gerrano *et al.*, 2015). Cultivation and consumption of *Amaranthus* is encouraged in Africa to alleviate malnutrition and micronutrient deficiencies (Achigan-Dako *et al.*, 2014).

1.2.3 Effect of climate change on weeds

In a world fraught with the serious impact of climate change, weeds are not immune to its effects. The main elements of climate change that will impact plants and weeds alike are changes in global temperatures and precipitation, increasing concentrations of atmospheric carbon dioxide (CO_2) and variations in frequency and intensity of climatic disasters (Scott *et al.*, 2014). Climate change is likely to exacerbate weed infestations and distribution as environmental conditions become more favourable to weeds (Padalia *et al.*, 2015).

Increasing temperatures allow plants to complete their lifecycle quicker resulting in weeds inhabiting areas they could not before, consequently extending their geographic range (Clements *et al.*, 2014). The vast majority of the world's problematic weeds are C_4 plants (Clements *et al.*, 2014). C_4 plants are able to photosynthesize more efficiently than C_3 plants at higher temperatures (Peters *et al.*, 2014). Consequently, these weeds may thrive as a result of elevated temperatures. Weeds also have the advantage of being more adept at utilising CO_2 (Clements *et al.*, 2014). Due to their high degree of genetic variation, some weeds may evolve successful morphological and physiological features more rapidly and outcompete crop plants (Peters *et al.*, 2014). However, the degree of competition is contingent on the plant species and the situation (Scott *et al.*, 2014).

1.3 IMPORTANT VECTOR-BORNE VIRUSES INFECTING TOMATO CROPS

1.3.1 *Potato virus Y* (PVY)

PVY is an important virus affecting potato (*Solanum tuberosum* L.) crops worldwide and has been the subject of intensive research over the years (Przewodowska *et al.,*

2015). PVY is the type member of the *Potyvirus* genus within the Potyviridae family (Kehoe and Jones, 2016). PVY isolates have diverse biological, serological and molecular characteristics thus leading to a complex classification of the virus (Janzac *et al.*, 2015). Historically PVY isolates were divided into three major groups, PVY^{O}, PVY^{N} and PVY^{C}. These were classified according to host phenotypic responses induced by PVY infections (Nie and Molen, 2015). Currently, nine strains have been identified, O, C, N, E, N-Wi, N:O, NTN, NA-N, and NE-11 (Goyer *et al.*, 2015). PVY infects a wide range of hosts including many solanaceous crops and some ornamentals. Among these are major economic crops such as potato, tomato (*Solanum lycopersicum* L), pepper (*Capsicum annuum* L.) and tobacco (*Nicotiana tabacum* L.) (Petrov and Gaur, 2015). PVY symptoms vary between mild mosaics to severe necrosis depending on the host and the strain of the virus (Fig 1.2a). Additional symptoms may include vein clearing, veinal necrosis, leaf deformation and stem necrosis (Goyer *et al.*, 2015). Necrotic patterns can also develop on tubers (Fig 1.2b; Petrov and Gaur, 2015). PVY is a single-stranded, monopartite, positive sense RNA virus, comprising of flexuous filamentous virions of 730-740x11-12 nm in size (Nerway, and Kassim, 2014; Kehoe and Jones, 2016). PVY is non-persistently transmitted by more than 40 aphid species with *Myzus persicae* being the most important given its wide distribution and transmission efficiency (Przewodowska *et al.,* 2015).

Figure 1.2: Mottling induced by PVY infection on foliage of potato plants (a) and necrotic lesions on tubers (b) (Petrov and Gaur, 2015; Kehoe and Jones, 2016)

1.3.2 *Tomato spotted wilt virus* (TSWV)

Arguably one of the most fascinating plant viruses, given its complex biology, is TSWV. It is among the most destructive plant viruses worldwide and was ranked as the second most important plant virus in a list compiled by *Molecular Plant Pathology* (Scholthof *et al.*, 2011). TSWV is the type member of the *Tospovirus* genus, the only plant virus genus, within the predominantly animal-infecting virus family, Bunyaviridae (Margaria *et al.*, 2014). The substantial economic impact of TSWV may be attributed to the extensive host range of TSWV. It possesses one of the largest host ranges, infecting more than 1000 species belonging to over 100 plant families (Margaria *et al.*, 2015). Furthermore what makes TSWV one of the most damaging plant viruses is its ability to break down the natural resistance of host plants (Margaria *et al.*, 2015). TSWV causes distinctive ringspots on fruit, foliage develop purple to black discolouration and leaf tip dieback is also observed on infected plants (Fig. 1.3; Zhang *et al.*, 2016). Virus transmission is mediated by thrips in a persistent propagative manner (Zhang *et al.*, 2016). Typical TSWV virions are spherical enveloped particles, with a diameter of 80-100nm (Ramesh and Pappu, 2016). Two glycoprotein projections, Gn and Gc, are displayed on the surface of virions (Rotenberg *et al*., 2015). TSWV is characterised by a negative and ambisense tripartite genome consisting of separately encapsidated linear single-stranded RNAs referred to as large (L), medium (M) and small (S) (Margaria and Rosa, 2015).

Figure 1.3: Leaf tip dieback on tomato leaves (a) and ringspots on tomato fruit (b) infected with TSWV (Riley *et al.*, 2011)

1.3.3 *Tomato yellow leaf curl virus* (TYLCV)

TYLCV has caused considerable economic losses since its emergence in the Middle East in 1931. Subsequently, it has been reported consistently in many tropical and sub-tropical areas (Kil *et al*, 2016). It is a whitefly-transmitted virus of the *Begomovirus* genus in the family Geminiviridae (Pakkianathan *et al*, 2015). Characteristic symptoms of TYLCV are yellowing, curling of leaves, stunting and in severe cases flowers abscise (Fig. 1.4; Pereira-Carvalho *et al.*, 2015). TYLCV is a monopartite single-stranded DNA virus encapsidated in germinate icosahedral virions (25-30nm; Pakkianathan *et al*, 2015). It is transmitted by several biotypes of the whitefly *Bemisia tabaci* in a circulative persistent manner, furthermore recent reports show that it is also seed transmissible (Ning *et al.*, 2015; *Kil et al*, 2016).

Figure 1.4: Typically leaf curling symptom of TYLCV on tomato plants (Hanssen *et al.*, 2010; Shirazi *et al.*, 2014)

1.3.4 *Tomato chlorosis virus* (ToCV)

ToCV is an emerging virus that poses a significant threat to crop production worldwide. It is the causal agent of yellow leaf disorder in tomato plants (Orílio *et al.*, 2014). ToCV is a member of the *Crinivirus* genus within the *Closteroviridae* family, where some of the largest RNA genomes of plant viruses are found (Landeo-Ríos *et al.*, 2015). It has a bipartite single-stranded RNA genome encapsidated in long flexuous virions approximately 800 to 850nm in length (Orílio *et al.*, 2014). ToCV naturally infects plants from eight different families, among those are the

economically important tomato, potato and sweet potato (*Ipomoea batatas* (L.) Lam) crops (Zhao *et al.*, 2014). Infected plants display interveinal chlorosis, leaf bronzing or brittleness (Fig. 1.5; Kil *et al.*, 2015a). Like with most *Criniviruses*, ToCV, is phloem limited and is not mechanically transmissible. It is semi-persistently transmitted by four species of whitefly, *B. tabaci*, *Bemisia argentifolii*, *Trialeurodes abutilonea* and *Trialeurodes vaporariorum* (Zhao *et al.*, 2014).

Figure 1.5: Interveinal chlorosis and leaf bronzing induced by ToCV infection on tomato plants (Barbosa *et al.*, 2011)

1.3.5 *Tomato torrado virus* (ToTV)

Tomato torrado virus (ToTV) is the type member of the genus *Torradovirus* within the family *Secoviridae* and is the causal agent of “Torrado disease” (Wieczorek *et al.*, 2015). The Torrado disease was first observed in tomatoes in Spain in 2001 but the causal agent was only identified in 2007 (Herrera-Vásqueza *et al.*, 2015). ToTV has since spread to Australia, Colombia, France, Hungary, Italy, Panama and Poland and has recently been reported for the first time in South Africa (Budziszewska *et al.*, 2016; Moodley *et al.*, 2016). Due to the virulence and the increasing global distribution of the virus, it is considered one of the most dangerous pathogens of tomato (Wieczorek and Obrępalska-Stęplowska, 2016). ToTV induces an initial symptom of chlorotic leaflet bases which subsequently develops into systemic necrosis of the entire plant including fruit (Fig. 1.6; Budziszewska *et al.*, 2016). ToTV has a bipartite single-stranded RNA genome and forms icosahedral virions that are

approximately 30nm in diameter (Herrera-Vásqueza *et al.*, 2015; Budziszewska *et al.*, 2016). The virus is semi-persistently transmitted by *B. tabaci* and *T. vaporariorum* (Wieczorek *et al.*, 2015).

Figure 1.6: Necrosis of tomato leaflet bases induced by ToTV infection (Wieczorek *et al.*, 2015)

1.4 IMPORTANT INSECT VECTORS OF PLANT VIRUSES INFECTING TOMATO CROPS

1.4.1 Whiteflies

Whiteflies (Hemiptera: Aleyrodidae) are among the most destructive agricultural pests globally, with more than 1500 species reported (Vásquez-Ordóñez *et al.*, 2015). They are typically found on the underside of leaves, where feeding and oviposition occur (Tosh and Brogan, 2015). The cosmopolitan sweet potato whitefly (*Bemisia tabaci*) is a prominent species that has garnered much attention due to its polyphagous nature, rapid propagation, enhanced vector competency and insecticide resistance (Fig. 1.7a; Chen *et al*., 2015). *B. tabaci* is a complex species of approximately 24 cryptic species (formerly known as biotypes) with extensive genetic variation (Fang *et al.*, 2013). Studies have shown that factors synthesised by whiteflies or the endosymbionts that inhabit them prevent the induction of plant defence responses when introduced to the host plant during feeding, thereby contributing to the high efficacy of virus transmission (Su *et al.*, 2015).

1.4.2 Aphids

Aphids (Hemiptera: Aphididae) are perhaps the best studied of all plant virus vectors (Roossinck, 2015). These small, ovate, soft-bodied phloem feeders are important

agricultural pests both as direct parasites to crops and as vectors of viral diseases (Warren and Schalau, 2014; Manicardi *et al.*, 2015). The green peach aphid (*Myzus persicae*) is the most significant aphid species given its polyphagous and ubiquitous nature (Fig. 1.7b; Przewodowska *et al.,* 2015). It has a vast host range, affecting more than 400 species within 40 different plant families (Bass *et al.*, 2014). Successful transmission of some aphid-borne viruses is dependent on more than simply the transmission of virions (Whitfield *et al.*, 2015). *Potyviruses* require the acquisition of additional viral proteins such as the helper component (HC) protein, along with the virions (Fereres and Raccah, 2015). Aphids exhibit sexual polymorphism. Depending on the environmental conditions, aphids switch between apomictic parthenogenesis (asexual) and sexual reproduction (Ogawa and Miura, 2014). Aphid infestations can occur rapidly due to the asexual stage in the life cycle of aphids, resulting in enhanced population growth (Jaouannet *et al.*, 2014).

1.4.3 Thrips

Thrips (order Thysanoptera) are minute, slender-winged, polyphagous insect pests responsible for the transmission of *Tospoviruses* (Montero-Astúa *et al*., 2014). A total of nine species of thrip transmit TSWV with the western flower thrip (*Frankliniella occidentalis*) being the most efficient vector (Fig. 1.7c; Rotenberg *et al*., 2015). Thrips acquire and spread the virus in a persistent, propagative manner (Seepiban *et al.*, 2015). Transmission and acquisition of the virus occur at specific stages during the thrip lifecycle. Both larvae and adult thrips can transmit the virus however, virus acquisition only occurs in the early larval stage (Riley *et al.*, 2011). Adult thrips may acquire the virus but they are not transmissible (Rotenberg *et al*., 2015). In addition to transmitting tospoviruses, thrips are economically important insect pests that can cause extensive feeding damage on host plants (Montero-Astúa *et al*., 2014).

Figure 1.7: Insect vectors of viral diseases: Sweet potato whitefly (*Bemisia tabaci*) (a), green peach aphids (*Myzus persicae*) (b) and western flower thrip (*Frankliniella occidentalis*) (c) (Riley *et al.*, 2011; Cuthbertson, 2013; Capinera, 2014)

1.5 ROLE OF WEEDS IN VIRUS EPIDEMIOLOGY

The complex ecological relationship between host, virus, vector and weed is an ancient one, however only in recent years has the role of weeds in viral ecology and epidemiology been the focus of investigations (Tahir *et al.*, 2015). Weeds are potential sources of primary viral inoculum of both known and unidentified virus species (Asala *et al.*, 2014). However, their impact was often overlooked in past studies (Prajapat *et al.*, 2014).

There have been an increasing number of reports of viruses detected on both known and unknown weed hosts in surrounding areas of crop fields (Kil *et al.*, 2015b; Leke *et al.*, 2015; Macharia *et al.*, 2016). Viruses are able to overwinter on weeds in the absence of the main host, allowing for the continual survival and spread of the virus in between cropping seasons (Kwon *et al.*, 2016). Some weed species are particularly important given their ability to host a variety of plant viruses thereby contributing to a high frequency of mixed infections on crops (Vafaei and Mahmoodi, 2015).

Weeds also act as reservoirs of viral vectors, most of which are polyphagous (Asala *et al.*, 2014). Furthermore, weeds can function as oviposition host of vectors, therefore allowing the build-up of vector populations during the offseason, and subsequent invasion of nearby crops and transmission of viruses (Macharia *et al.*, 2016). Some vectors have been known to probe into leaf surfaces regardless of whether the plant is a host and as a result may transmit viruses (Jaouannet *et al.*, 2014). This behaviour is said to contribute to the high transmission rates of viruses on non-host plants.

It has been reported that some vectors are capable of transmitting viruses more efficiently from infected weeds to nearby crops than crop to crop transmission (Srinivasan *et al.*, 2013). Studies have shown that some insect vectors have a predilection for weed hosts. As previously reported by Srinivasan *et al.* (2013) green peach aphids had a greater preference for *Solanum sarrachoides* (hairy nightshade) than the host potato crop, consequently enhancing vector fecundity and longevity. Further research showed that TYLCV infection on *D. stramonium* improved vector fitness and attraction to weed hosts (Chen *et al.*, 2013). This implies that vectors perform better on wild plants infected with viruses.

Virus infection induces volatile organic compounds (VOCs) in plants which modulate insect vector attraction (Vafaei and Mahmoodi, 2015). Non-persistently transmitted viruses induced anti-feeding compounds which caused the rapid movement of vectors onto the next crop after virus acquisition (Carmo-Sousa *et al*., 2014). Contrastingly pro-feeding behaviour was prompted by persistently transmitted viruses (Roossinck, 2015). This phenomenon induced by the virus infection optimises the efficacy of viral spread and transmission.

1.6 DETECTION OF VIRUSES

Plant viruses are notoriously difficult to detect and identify. Traditional disease diagnosis methods involve assessing disease symptomology, however, this tactic alone is not a reliable method of detection as many plant viruses elicit similar symptoms on host plants or plants are asymptomatic (Alemu, 2015). Currently, there are no direct control measures of plant viruses, therefore early detection and accurate disease diagnosis are imperative to prevent crop loss (Jeong *et al.*, 2014).

Serological tests based on the interaction of an antibody and antigen is a popular tool in the diagnosis of plant viral diseases. This approach of enzyme-linked immunosorbent assay (ELISA) transformed the application of serology by its simplicity, specificity and high throughput potential (Alemu, 2015). Given its frequent use, ELISA has become established as the global standard tool in viral diagnostics (Boonham *et al.*, 2014). A drawback of ELISA is that the antisera used in tests lack the ability to correctly identify virus strains (Jeong *et al.*, 2014). Furthermore, it is unable to detect the virus when a low titre of antiserum is used (Kwon *et al.*, 2013).

Nucleic acid-based detection methods are highly sensitive techniques used in screening and identification of viruses by targeting any region of a viral genome (Kwon *et al.*, 2013). Polymerase chain reaction (PCR), a powerful tool in viral diagnostics, is able to detect trace amounts of viral DNA in plants (Alemu, 2015). Modified PCR methods such as reverse transcription – PCR (RT-PCR) is used to detect positive sense RNA viruses by transcribing viral RNA in copied DNA (cDNA) (Patel *et al.*, 2016). PCR techniques are commonly used in diagnostic laboratories and in molecular experiments (Jeong *et al.*, 2014).

The innovative next generation sequencing (NGS) platforms offer a comparatively high through-put potential to previous single-analyte microarrays (Lefterova *et al.*, 2015). In contrast to traditional sequencing platforms which measured signal intensities, NGS digitally counts sequencing reads allowing for a more sensitive quantification and detection of gene expression and nucleic acids (Anonymous, 2015). Commercially there are several NGS platforms available including Roche's 454, Illumina's Genome Analyzer, ABI's SOLiD and the Heliscope from Helicos (Magi *et al.*, 2010). Samples are initially prepared by extraction of nucleic acid (Fig. 1.8A). A library of fragmented nucleic acids is prepared and adapters are subsequently added on the fragmented ends (Fig. 1.8B), allowing for efficient parallel sequencing of the short nucleic acid segments (Fig. 1.8C; Anonymous, 2015). The short reads are aligned and reassembled according to a reference genome resulting in the complete sequence of the original nucleic acid (Fig. 1.8D and 1.8E; Roy *et al.*, 2016).

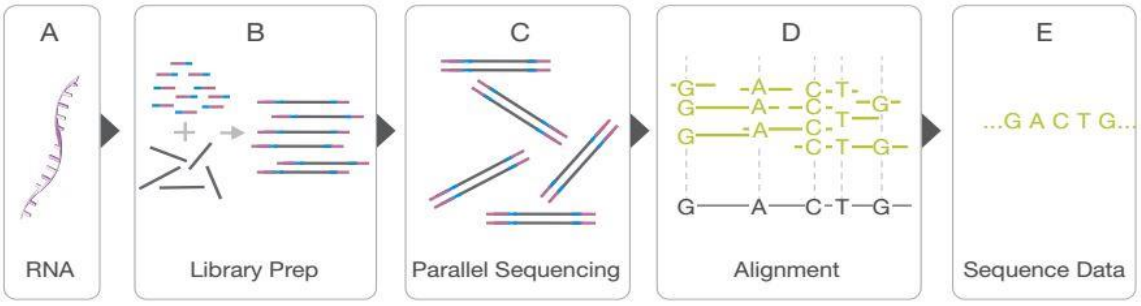

Figure 1.8: Schematic representation of Next-generation sequencing (NGS) (Anonymous, 2015)

1.7 CONTROL OF WEEDS IN AGRICULTURE

Historically, conventional tillage was the primary control strategy used to manage weeds in agriculture (Ajirloo and Ahangar, 2014). Consequently, this practice significantly destroyed soil structure leading to the collapse of soil profiles (Carbonetto *et al.*, 2014). This paved the way for the era of conservation agriculture where there is minimal soil disturbance (Nicholsa *et al.*, 2015). However, the major drawback of conservation agriculture is weed management (Santín-Montanyá *et al.*, 2016). This is particularly challenging for resource-poor smallholder farmers in sub-Saharan Africa where weed control is labour intensive and expensive (Mhlanga *et al.*, 2016).

Conservation agriculture contributed to the over-reliance on synthetic herbicides (Nicholsa *et al.*, 2015). This has led to unprecedented artificial selection pressure on weed populations causing the prevalence of herbicide-resistant “superweeds” (Clements *et al.*, 2014). Currently, certain weed populations have developed a resistance to 23 of the 26 herbicide target sites and to 160 different herbicides (Heap, 2016).

Potential strategies proposed to ameliorate the problem of weed management is by biological means and the use of cover crops among other cultural practices. Fungal and bacterial biocontrol agents have garnered much attention in the last several decades. The prominent fungal and bacterial genera used as potential bioherbicide agents comprise of *Colletotrichum*, *Phoma*, *Sclerotinia*, *Xanthomonas* and *Pseudomonas* (Harding and. Raizada, 2015). Interestingly, viruses of weeds have been examined as possible bioherbicide agents (Diaz *et al.*, 2014). *Tobacco Mild Green Mosaic Tobamovirus* has been approved for use to control tropical soda apple (*Solanum viarum*) in Florida (EPA, 2015). Cover crops are an inexpensive alternative to herbicides for resource-poor farmers. It provides sufficient management of weeds particularly when used in rotation with the main crop, however, careful follow-up is required throughout the proposed period cover crops will be in use. (Lemessa and Wakjira, 2015). Some cover crops have an allelopathic effect on certain weed species thereby reducing the presence of weeds in crop fields (Mhlanga *et al.*, 2016).

1.8 CONCLUSION

The impact of weeds on the ecology and epidemiology of plant infecting viruses cannot be overemphasised. Weeds harbouring both viruses and the vectors that transmit them ensures a persistent reservoir of vectors and vector-borne diseases in the vicinity of crop fields. Since there are no direct control measures of plant viruses, the elimination of alternate weed hosts is crucial. It is imperative that stringent cultural control practices, part of an integrated pest management programme, be employed to reduce the impact of weeds on the incidence of vector-borne diseases.

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CHAPTER TWO

IDENTIFICATION AND DISTRIBUTION OF WEEDS HARBOURING VIRAL DISEASES IN SOUTH AFRICA[1]

2.1 ABSTRACT

The relationship between host, virus and vector is a complex and ancient one. The role of weeds as reservoirs of viral pathogens has significantly impacted on viral epidemiology in many parts of the world including South Africa. Weeds act as alternate hosts for a number of viruses and the polyphagous vectors that transmit them. Only recently has the role of weeds in viral epidemiology and ecology been the focus of investigation. While there is an increasing number of reports on viruses identified on weeds globally, there is a significant lack of information on the distribution of weeds and the viruses they harbour in South Africa. The aim of this study was to identify important viruses infecting weed species in South Africa. A nationwide survey was conducted in major tomato growing areas to identify weed species that harbour vectors and vector-borne viruses. Fifteen weeds species growing near and within tomato crop fields were found exhibiting virus-like symptoms in the presence of excessively high aphid, thrip and whitefly populations. Symptoms ranged from mosaics, mottling, necrotic spots, leaf curling, interveinal chlorosis and purple discoloration of leaves. Similar symptoms were also observed on tomato crops in fields. *Tomato spotted wilt virus*, *Potato virus Y*, *Tomato torrado virus* and *Tomato chlorosis virus* were positively identified using reverse transcription polymerase chain reaction (RT-PCR) and confirmed using BLAST, on *Amaranthus thunbergii*, *Physalis peruviana, Datura stramonium* and *Solanum nigrum* respectively. These viruses were also detected on tomato crops. These results

[1] Parts of this research project was published in:

1. Kisten, L. Moodley, V., Gubba, A. and Mafongoya, P.L. 2016. First report of *Potato virus Y* (PVY) on *Physalis peruviana* in South Africa. *Plant Disease* 100(7):1511.
2. Kisten, L. Moodley, V., Gubba, A. and Mafongoya, P.L. 2016. First detection of *Tomato spotted wilt virus* (TSWV) on *Amaranthus thunbergii* in South Africa. *Plant Disease* 100(10):2176.
3. Kisten, L. Moodley, V., Gubba, A. and Mafongoya, P.L. 2016. Noxious weeds, reservoirs of vector-borne viral pathogens in South Africa. Indian Phytopathological Society 6th International Conference on “Plant, Pathogens and People”. New Delhi, India. ISBN: 81-7019-527-2.

confirm that weeds harbour viral pathogens which cause serious economic losses to crop production. The density and distribution of weeds in South Africa emphasise their role in viral epidemiology. Stringent weed control methods such as crop husbandry, herbicide application and scouting can be collectively employed to reduce vector-borne viral diseases.

2.2 INTRODUCTION

Tomato (*Solanum lycopersicum* L.), a member of the cosmopolitan Solanaceae family, is a widely cultivated vegetable crop worldwide (Mueller *et al.*, 2005). It is the second most important vegetable crop after potatoes (Olaniyi *et al.*, 2010). In 2010, global tomato production reached 5 million ha with a total harvest of more than 129 million tonnes (Srinivasan, 2010). In South Africa, the tomato industry with an annual production of 566 180 tonnes is relatively small when compared to that of the world leading producer China, which has an annual production of 50 552 200 tonnes (FAOSTAT, 2016). Tomatoes are an important condiment in daily human nutrition and are an affordable source of vitamins and minerals (Srinivasan, 2010). In addition to their nutritional value, tomatoes contain high levels of lycopene, an antioxidant that reduces the risk of cancer and some neurodegenerative diseases (Olaniyi *et al.*, 2010).

Plant viruses are a major constraint to crop production worldwide. Crop losses due to viral diseases in developing countries, whose economies are primarily agriculturally based, have serious food security and socio-economic implications. This holds true for resource poor, subsistence farmers for whom crops are important commodities as income and food sources (Nono-Womdim, 2003). In Africa, viral diseases cause significant economic losses, second only to fungal diseases (Otim-Napea *et al.*, 2003).

The tomato crop is susceptible to a considerable number of phytopathogens, vector-borne viral diseases, in particular, many of which are ubiquitous and have broad host ranges. Approximately 136 viruses are known to infect tomato crops (Hanssen *et al.*, 2010). The most notable vector-borne viruses affecting tomato crops are *Tomato spotted wilt virus* (TSWV), *Potato virus Y* (PVY), *Tomato yellow leaf curl virus* (TYLCV) and the emerging *Tomato chlorosis virus* (ToCV) and *Tomato torrado virus*

(ToTV) (Verbeek *et al*., 2007; Hanssen *et al.*, 2010; Rybicki, 2015). Collectively these viruses have serious repercussions for tomato production globally.

It is widely known that weeds serve as alternate hosts of viral diseases, however, research on the impact this has on viral ecology and epidemiology is often neglected (Mubin *et al.*, 2009). Weeds form part of an ancient and exceedingly complex interaction between host plants, viruses, insect vectors and agro-climatic conditions (Sastry and Zitter, 2014). Weeds play an integral role in virus epidemiology as reservoirs of viral pathogens and by acting as initial sources of viral inoculum (Thresh, 2003). Moreover, weeds also harbour insect vectors that are responsible for viral transmission, resulting in rapid vector population growth (Wisler and Norris, 2005). Consequently, viral disease incidence persists within crop fields and epidemics may occur more frequently (Sastry and Zitter, 2014).

Effective virus management should necessarily encompass the accurate identification of weed hosts harbouring viruses together with the viruses infecting these weeds. The removal of weeds is still often neglected in agroecosystems, particularly along areas surrounding crop fields. Consequently, this provides initial sources of viral infections which can be transmitted by insect vectors into neighbouring field crops. With virological research now shifting to encompass the study of weeds, there are increasing reports of specific viral infections on weed species. In South Africa, however, there are few official reports of virus infections on weeds. Therefore the aim of this study was to provide comprehensive information on the identity and distribution of important viruses infecting weed species in South Africa. Information generated from this study will contribute towards developing comprehensive and effective management strategies to control viruses in agricultural settings.

2.3 MATERIALS AND METHODS

2.3.1 Field survey

A field survey to identify viruses infecting weeds was conducted between 2015 and 2016 in major tomato growing regions in the nine provinces of South Africa. The survey areas included both large commercial and small-scale farms that cultivated predominantly tomato crops as well as other Solanaceae crops on a smaller scale

(Fig. 2.1). A total of 24 weeds displaying virus-like symptoms including 15 different weed species representing six plant families were randomly sampled. Infected weeds had high vector populations on leaf surfaces. Large whiteflies populations, in particular, were often observed. These samples were obtained from 33 crop fields during the survey (Fig. 2.1). The sample site map was constructed using ArcGIS v9.3 (ESIR, 2011).

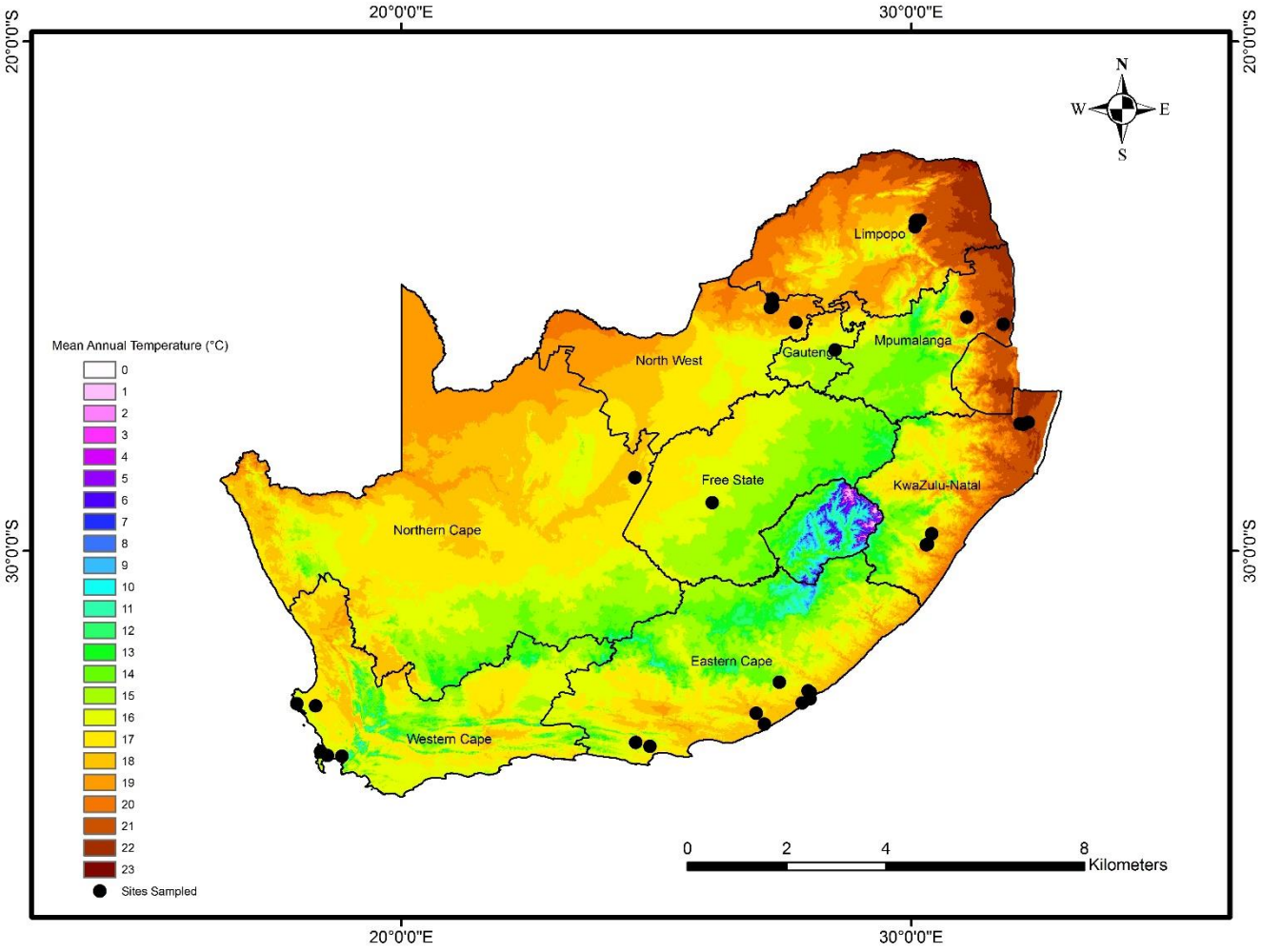

Figure 2.1: Map of South Africa depicting the locations of tomato production areas sampled during the field survey

2.3.2 Identification of weed species sampled

The weed species sampled during the survey were identified using textbooks (Grabandt, 1985; Henderson, 2001; Bromilow, 2010) and by consulting herbarium

specimens at The Bews Herbarium situated at the University of KwaZulu-Natal (UKZN-PMB).

2.3.3 Nucleic acid extraction

Total RNA and DNA were extracted from symptomatic weed leaf material using the Quick-RNA™ MiniPrep Kit (Zymo Research, USA) and Quick-DNA™ Universal Kit (Zymo Research, USA), respectively according to manufacturers' instructions. Plant tissue was homogenised using the Mini-beadbeater (BioSpec, USA). RNA and DNA preparations were stored at -80°C and -20°C respectively until analysed.

2.3.4 RT-PCR, PCR of total nucleic acid and *Crinivirus* multiplex RT-PCR

Total RNA from infected leaf material was used as a template for first strand synthesis in the reverse transcription (RT) protocol. RT was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to manufacturers' instructions. cDNA was synthesised from total RNA samples using the degenerate reverse primer of *Potyvirus*, *Torradovirus, Tospovirus* and *Crinivirus* (Solanaceae) and the *Potato virus Y* specific reverse primer (Table 2.1) in 20µl reactions. The tubes were incubated for 1 hour at 42°C on a G-Storm thermal cycler followed by 5 minutes of heating at 70°C to terminate the reaction.

Weed samples were also tested for the presence of DNA viruses using a universal *Begomovirus* primer (Table 2.1) in addition to the aforementioned RNA viruses. The PCR and the multiplex PCR reactions were performed in a 20µL volume with 3 µl of cDNA using KAPA2G Fast DNA Polymerase (Kapa Biosystems, South Africa) according to the manufacturers' instructions. Amplification was performed in the G-Storm thermal cycler programmed according to the following parameters. The reaction mix was heated at 95°C for 2 minutes to activate the polymerase enzyme. This was followed by 35 cycles of amplification with 25s of denaturation at 95°C, 20s of annealing at respective temperatures for each primer pair (Table 2.1), and 20s of elongation at 72°C followed by a final extension for 5 minutes at 72°C.

PCR amplicons were examined by electrophoresis on 1.5% (w/v) agarose gels stained with SYBR® safe (Invitrogen, USA). Positive PCR amplicons were purified using the QIAquick® Gel Extraction Kit (Qiagen, Germany) according to

manufacturers' instructions and were directly sequenced in both directions at Inqaba Biotec (Inqaba Biotechnical Industries, Pretoria, South Africa).

2.3.5 ELISA

2.3.5.1 TSWV

Symptomatic leaf tissue from 24 weeds and 40 tomato samples were tested with TSWV-specific antibodies (Neogen, Europe) using DAS-ELISA to detect the presence of TSWV. Samples were tested according to the manufacturers' instructions. Samples were analysed by reading the absorbance at 405nm on a FLUOstar OPTIMA microplate reader (BMG Labtech, Germany) and visual assessment for varying degrees of yellow colour development. Absorbance values three times that of the negative control absorbance values were considered a positive reaction.

2.3.5.2 PVY

ELISA was used to test the presence of PVY and to determine the specific PVY strain on infected samples. Symptomatic leaf tissue sampled during the survey were tested with PVY^{N}, $PVY^{O/C}$ and PVY^{O} antibodies (Neogen, Europe) using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA). To test leaf samples using DAS-ELISA and TAS-ELISA, the protocol was followed according to the manufacturers' instructions. Samples were analysed by reading the absorbance at 405nm on a FLUOstar OPTIMA microplate reader (BMG Labtech, Germany). Absorbance values three times that of the negative control absorbance values were considered a positive reaction.

2.3.6 Phylogenetic analysis

The amplified nucleotide sequences were analysed and aligned using the Molecular Evolutionary Genetics Analysis (MEGA 6; Tamura *et al.*, 2013) software and Clustal W. The virus isolates detected in this study were analysed by comparison with sequence data from known parental isolates selected from the public database of the National Centre for Biotechnology Information (NCBI) (www.ncbi.nml.nih.gov) using the BLAST programme (Table 2.5). The sequence data reported in this research were deposited into GenBank. The phylogenetic trees were inferred using the

Table 2.1: Universal primers used for amplification of *Begomovirus*, *Crinivirus, Potyvirus*, *Torradovirus* and *Tospovirus*

VIRUS	PRIMERS	SEQUENCES 5' – 3'	AMPLICON	ANNEALING TEMPERATURE	TARGET REGION	REFERENCE
Begomovirus	TY1(+)	GCCCATGTA(T/C)-CG(A/G)AAGCC	580 bp	50°C	Coat protein	Accotto *et al.*, 2000
	TY2(-)	GG(A/G)TTAGA(A/G)GCATG(A/C)GTAC				
Crinivirus	Solanaceae (R)[c]	GTGTTBGAYAACCAWGTGTT		52°C	RNA-dependent RNA polymerase	Wintermantel and Hladky, 2010
	TICV (F)[c]	AAGAATGGACCTACCCAG	995 bp			
	ToCV (F)[c]	GCACCCTGATTGGTTCTAAAC	265 bp			
	PYVV (F)[c]	ATCGTTCGTTCTCAACCG	514 bp			
Potyvirus	M4T (R)[a]	GTT TTCCCAGTCACGAC$(T)_{15}$	1700 bp	47°C	Nuclear inclusion body b	Chen *et al.*, 2001
	S-primer[b]	GGXAAYAAYAGYGGXCAZCC				
	M4[b]	GTTTTC CCAGTCACGAC				
Potato virus Y	VPg-R	GCTTCATGYTCYACHTCCTG	547 bp	56°C	Viral genome–linked protein	Ben Khalifa *et al.*, 2009
	VPg-F	GAATYCAAGCHYTRAAGTTTCG				
Torradovirus	Torrado-1R	GGWACWGCMACHAGRTTGTCATC	371 bp	52°C	RNA-dependent RNA polymerase	Verbeek *et al.*, 2012
	Torrado-1F	GCWGAYTAYTCMAGYTTTGATGG				
	Torrado-2R	CCWGTCCACCAYTTGCAATT	515 bp	52°C	Coat proteins Vp35 and Vp26	Verbeek *et al.*, 2012
	Torrado-2F	TGGGATGARTGYAATGTKCT				
Tospovirus	gM870c (R)	ATTAGYTTGCAKGCTTCAATNAARGC	500 bp	52°C	Non-structural protein on M strand	Chen *et al.*, 2012
	gM410 (F)	AACTGGAAAAATGATTYNYTTGTTGG				

[a] Initial reverse Potyviridae primer used to synthesise cDNA in RT reaction
[b] Potyviridae amplification primers used in PCR reaction
[c] Crinivirus primers used in a multiplex RT-PCR

Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) and the Jukes-Cantor model (Jukes and Cantor, 1969). The bootstrap analysis was conducted using 1000 replicates. The phyloegentic trees were rooted using *Bean yellow mosaic virus* (BYMV), *Beet pseudoyellows virus* (BPYV), *Tomato marchitez virus* (ToMarV) and *Groundnut ring spot virus* (GRSV) as outgroups.

2.4 RESULTS

2.4.1 Field assessment

The weed samples collected from within and on the edges of tomato fields exhibited a diverse array of symptoms characteristic of virus infection. Symptoms ranged from mosaics, mottling, necrotic spots, leaf curling, interveinal chlorosis and purple discoloration of leaves (Fig. 2.2). Interestingly, these symptoms were also observed on nearby tomato crops.

2.4.2 RT-PCR, PCR and multiplex RT-PCR

RT-PCR amplicons of expected band sizes were obtained using universal *Tospovirus*, *Torradovirus* and *Potyvirus* primers as well as the virus-specific PVY primers on *A. thunbergii*, *D. stramonium* and *P. peruviana* respectively (Table 2.2). The Crinivirus multiplex RT-PCR showed positive PCR amplicons with the ToCV specific primers on *D. stramonium* and *S. nigrum* (Table 2.2). BLAST analysis of the isolates confirmed the detection of PVY, TSWV and ToCV on *P. peruviana*, *A. thunbergii* and *S. nigrum* respectively (Table 2.2). Moreover, BLAST analysis showed that the PVY isolate most closely matched to a PVY^{C} strain (Table 2.3), hence further analysis was done to determine the strain of the PVY isolate. A mixed infection of ToTV and ToCV was detected on a *D. stramonium* isolate (Table 2.2). Similarly, PVY, TSWV, ToTV and ToCV were also detected on tomato crops from the same sample sites of the weeds (data not shown). TSWV was also detected in a tomato sample that was collected a significant distance from the weed sample site, approximately 840km away (GenBank accession no. KY039275; Table 2.3).

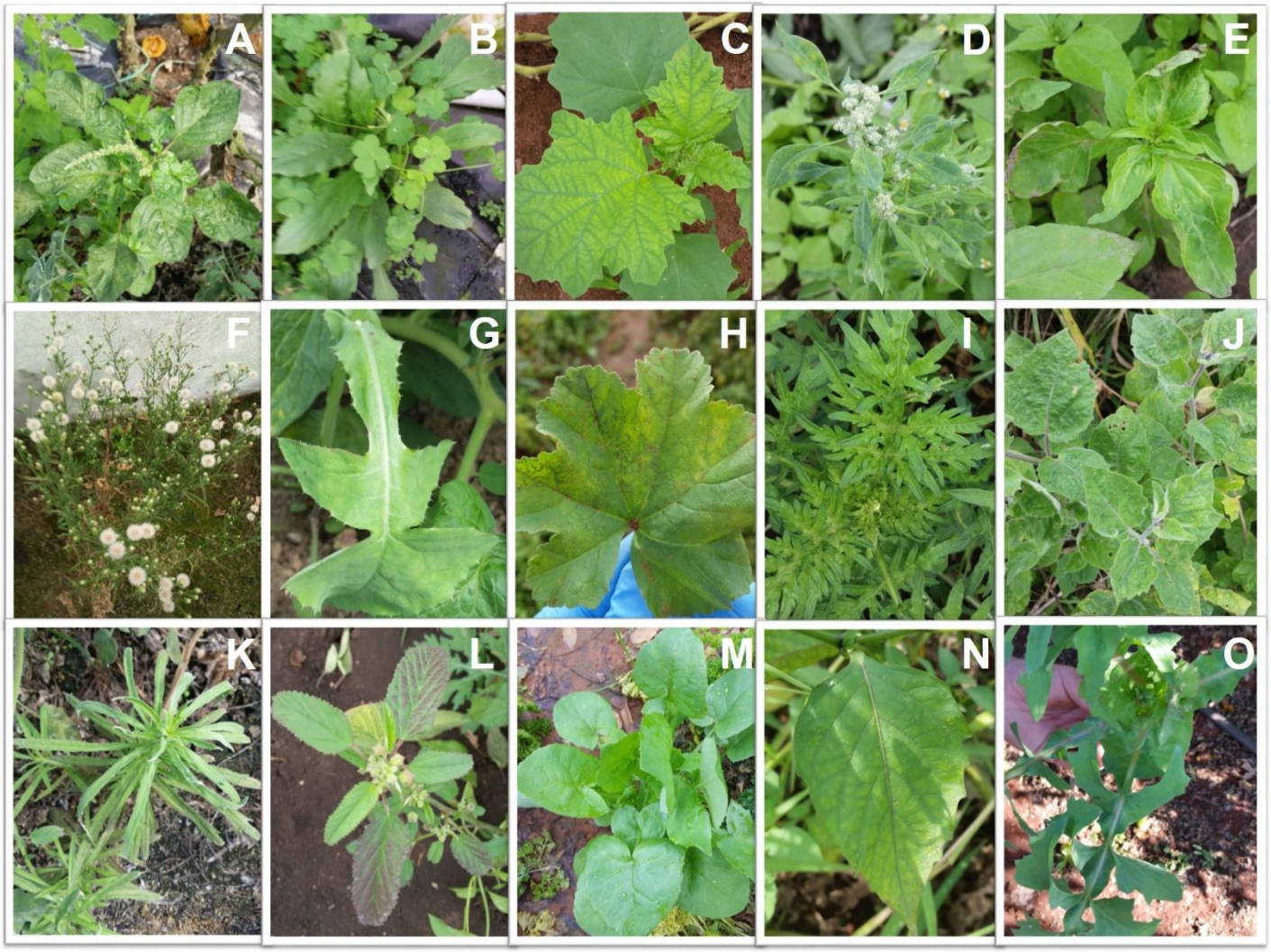

Figure 2.2: Symptoms observed on weed species sampled in and around tomato crop fields in South Africa. (A) *Amaranthus thunbergii,* (B) *Conyza bonariensis,* (C) *Datura stramonium*, (D) *Euphorbia* sp., (E) *Galinsoga parviflora,* (F) *Gamochaeta pensylvanica,* (G) *Lactuca* sp., (H) *Malva parviflora,* (I) *Parthenium hysterophorus,* (J) *Physalis peruviana,* (K) *Senecio polyanthemoides,* (L) *Sida cordifolia,* (M) *Sisymbrium* sp., (N) *Solanum nigrum,* (O) *Sonchus oleraceus.*

Table 2.2: Weed samples tested for PVY, ToCV, ToTV, TSWV and TYLCV infection using RT-PCR, PCR and multiplex RT-PCR

WEED FAMILY	WEED SPECIES	NO. OF SAMPLES	NO. OF SAMPLES POSITIVE	LOCATION	VIRUS SPECIES					GENBANK ACCESSION NUMBER
					PVY	ToCV	ToTV	TSWV	TYLCV	
Amaranthaceae	*Amaranthus thunbergii*	2	1	Port Elizabeth	-	-	-	+	-	KU975371
Asteraceae	*Conyza bonariensis*	1	-	Bloemfontein	-	-	-	-	-	
	Galinsoga parviflora	2	-	Jozini, Nelspruit	-	-	-	-	-	
	Gamochaeta pensylvanica	1	-	Brits	-	-	-	-	-	
	Lactuca sp.	2	-	Barkly West,	-	-	-	-	-	
	Parthenium hysterophorus[c]	1	-	Jozini	-	-	-	-	-	
	Senecio polyanthemoides	2	-	East London	-	-	-	-	-	
	Sonchus oleraceus	1	-	Mogwase	-	-	-	-	-	
Brassicaceae	*Sisymbrium* sp.	1	-	Pretoria	-	-	-	-	-	
Euphorbiaceae	*Euphorbia* sp.	2	-	Saldanha Bay	-	-	-	-	-	
Malvaceae	*Malva parviflora*	1	-	East London	-	-	-	-	-	
	Sida cordifolia	1	-	East London	-	-	-	-	-	
Solanaceae	*Datura stramonium*[bc]	3	1	Port Elizabeth, Pretoria, Mooketsi	-	+	+	-	-	KT989862[a] & KP890356
	Physalis peruviana	3	1	Pietermaritzburg	+	-	-	-	-	KU236384
	Solanum nigrum	1	1	Nelspruit	-	+	-	-	-	KT989862[a]

(+) Positive PCR amplicon obtained (-) No band observed at the expected band size

[a] ToCV sequence data deposited in GenBank as a consensus sequence [b] Mixed infection of ToCV and ToTV

[c] Ranked as a category 1b invasive species according to the South African National Environmental Management: Biodiversity Act (NEMBA).

Table 2.3: BLAST analysis of virus isolates detected in this study

SAMPLE	% IDENTITY MATCH	CLOSEST ISOLATE MATCH	ORIGIN	GENBANK ACCESSION NUMBER	REFERENCES
A. thunbergii	91% to TSWV	TSWV-FG31	Italy	AY124963	Finetti Sialer *et al.*, 2002
D. stramonium	99% to ToTV	Wal'03	Poland	EU563947	Budziszewska *et al*., 2008
	98% to ToCV	Tenerife	Spain	KJ175084	Fiallo-Olivé *et al.*, 2014
P. peruviana	85% to PVY	PVY JVW-186	South Africa	KF770835	Moodley *et al.*, 2014
S. nigrum	98% to ToCV	Tenerife	Spain	KJ175084	Fiallo-Olivé *et al.*, 2014
Tomato	91% to TSWV	TSWV-FG31	Italy	AY124963	Finetti Sialer *et al.*, 2002

2.4.3 DAS-ELISA and TAS-ELISA of TSWV and PVY

P. peruviana had the highest DAS-ELISA absorbance value with the $PVY^{O/C}$ antiserum, indicating the possibility of either an O or C strain (Table 2.4). Therefore TAS-ELISA was done and *P. peruviana* had the highest TAS-ELISA absorbance value with the PVY^{O} antiserum. *A. thunbergii* and one tomato isolate showed a positive reaction for TSWV (Table 2.4). These values were similar to the values obtained for the positive controls (Table 2.4). The results corresponded to the visual assessment of the yellow colour development on positive samples (data not shown). These results indicate the presence of PVY on *P. peruviana* and TSWV on *A. thunbergii* and tomato. Furthermore, it suggests the possibility of the presence of PVY^{O} on *P. peruviana*.

Table 2.4: Serological detection of TSWV and the PVY strain on *A. thunbergii* and *P. peruviana* respectively using DAS-ELISA and TAS-ELISA and quantification of viral load

SAMPLES	DAS-ELISA		TAS-ELISA	
	PVY^{N}	TSWV	$PVY^{O/C}$	PVY^{O}
Positive control	0.3142	1.034	0.3445	1.1607
Negative control	0.1885	0.531	0.1685	0.3319
Sample - *P. peruviana*	0.1524	-	0.3522	1.6250
Sample - *A. thunbergii*	-	1.517	-	-
Sample - Tomato	-	1.506	-	-

2.4.4 Phylogenetic analysis

The nucleotide sequences of the RNA-dependent RNA polymerase (RdRp), viral genome–linked protein (VPg), coat protein (CP) and non-structural protein on M strand (NSm) of ToCV, PVY, ToTV and TSWV respectively detected in this study were aligned with the respective protein sequences of each virus from different geographical origins (Table 2.5). Details of the isolates used in the phylogenetic analysis are given (Table 2.5).

The sequence of the PVY isolate did not display any genetic diversity and clustered within an African subgroup of the PVY lineage (Fig. 2.3). The sequence of the ToCV isolate clustered with South Korean isolates (Fig. 4). In contrast, the sequences of the ToTV and both TSWV isolates did not cluster with any of the parental ToTV and TSWV isolates. These sequences displayed genetic diversity among other isolates and formed a distinct separate clade (Fig 2.5; 2.6). Phylogenetic analysis of the TSWV isolates (TOSP-186 and LAV10) coupled with the high bootstrap percentage value suggests that these isolates are the same (Fig. 2.6).

Table 2.5: Isolates obtained from the NCBI nucleotide database used for phylogenetic analysis in conjunction with isolates from this study

VIRUS	ISOLATE	ORIGIN	ACCESSION NO.	REFERENCE
PVY	UKU-VP_Vpg-R	South Africa	KU236384	This study
	JVW-186	South Africa	KF770835	Moodley *et al.*, 2014
	Arka	Poland	EF638905	Kosakowski *et al.*, 2007
	PO27	France	KF670579	Moury *et al.*, 2014
	K4-11-94	Tunisia	KF670595	Moury *et al.*, 2014
	Pepper-Zim1	Zimbabwe	KU695465	Karavina *et al.*, 2016
	YCZb	Poland	JF804797	Golnik and Szyndel, 2011
	PRI-509	Netherlands	EU563512	Dullemans *et al.*, 2011
BYMV	LutKP	Australia	AY376314	Unpublished
ToCV	Nel-186Cr	South Africa	KT989862	This study
	Tenerife	Spain	KJ175084	Fiallo-Olivé *et al.*, 2014
	Sudan 3	Sudan	JN411686	Fiallo-Olivé *et al.*, 2014
	PI-1-2	Spain	KJ200308	Villanueva *et al.*, 2014
	MM8	Spain	KJ200306	Villanueva *et al.*, 2014
	AT80/99-1C	Spain	KJ740256	Orílio *et al.*, 2014
	JJ	South Korea	KP137100	Lee *et al.*, 2014
	HS	South Korea	KP137098	Lee *et al.*, 2014
BPYV	OZ2	Japan	LC100134	Unpublished
ToTV	Lim-186	South Africa	KP890356	This study
	Wal'03	Poland	EU563947	Budziszewska *et al.*, 2008
	sec2	Colombia	KJ571199	Angel-Diaz and Martinez-Henao, 2014
	Ros	Poland	KM114266	Budziszewska *et al.*, 2016
	Kra	Poland	KJ940974	Budziszewska *et al.*, 2016
	sec3	Colombia	KJ571200	Angel-Diaz and Martinez-Henao, 2014
	sec1	Colombia	KJ571198	Angel-Diaz and Martinez-Henao, 2014

VIRUS	ISOLATE	ORIGIN	ACCESSION NO.	REFERENCE
ToMarV	LV2011GveTe8	Mexico	KF267244	Unpublished
TSWV	TOSP-186	South Africa	KU975371	This study
	LAV10	South Africa	KY039275	This study
TSWV	Cr1NL2	Spain	HM015511	López *et al.*, 2011
	TSWV-CEC1	Italy	AY124950	Finetti Sialer *et al.*, 2002
	NC-3	USA	AY744486	Tsompana *et al.*, 2005
	Ber1TL3	Spain	HM015517	López *et al.*, 2011
	p202/3RB	Italy	HQ830185	Margaria *et al.*, 2014
	D M	USA	AF208497	Hoffmann *et al.*, 2001
	TSWV-FG31	Italy	AY124963	Finetti Sialer *et al.*, 2002
	TSWV-32-1	Italy	AY124960	Finetti Sialer *et al.*, 2002
	L3	Bulgaria	X93603	Soellick *et al.*, 2000
	158-Gerb	Serbia	HQ246453	Stanković *et al.*, 2011
	33-06	Serbia	GQ373174	Bulajić *et al.*, 2014
	53-05	Serbia	GQ373176	Bulajić *et al.*, 2014
GRSV	GRSV-AR	Argentina	KT972591	De Breuil *et al.*, 2016

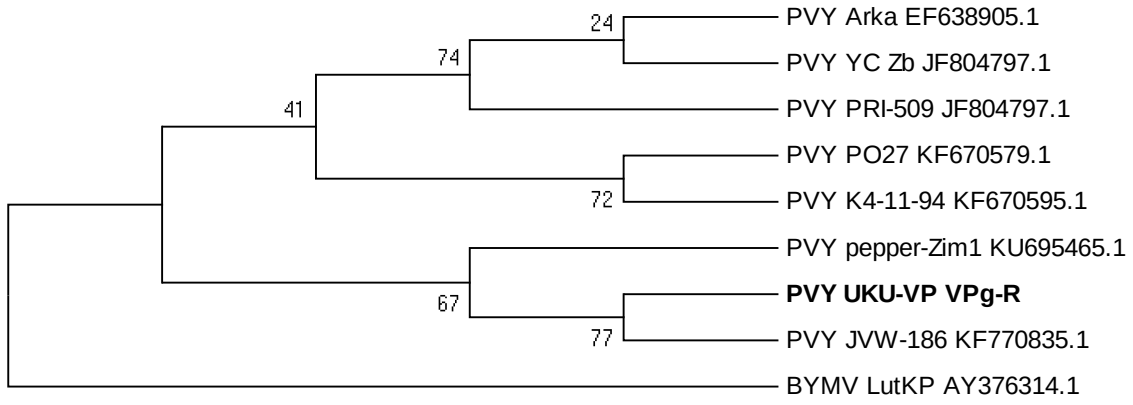

Figure 2.3: Phylogenetic relationship of the sequence of the PVY isolate from South Africa

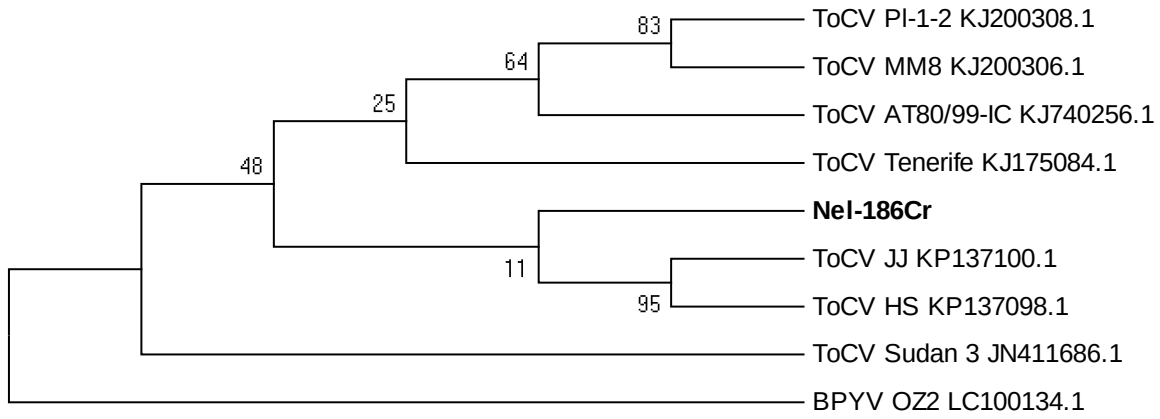

Figure 2.4: Phylogenetic relationship of the sequence of the ToCV isolate from South Africa

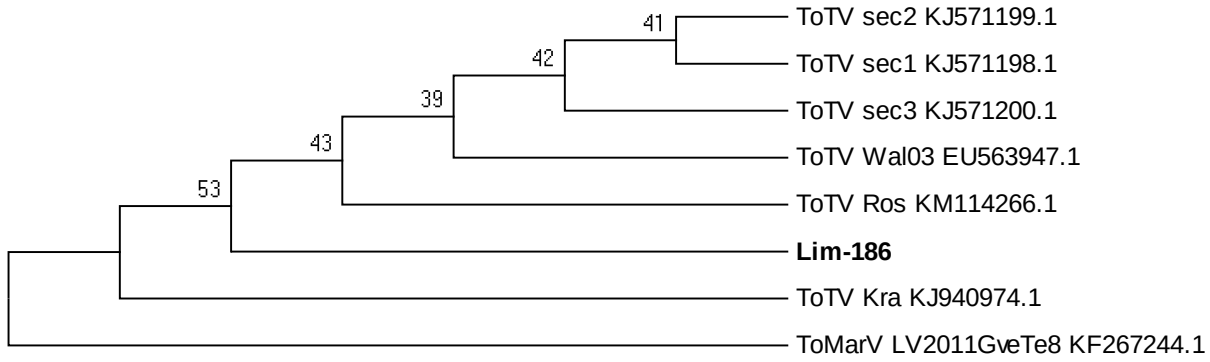

Figure 2.5: Phylogenetic relationship of the sequence of the ToTV isolate from South Africa

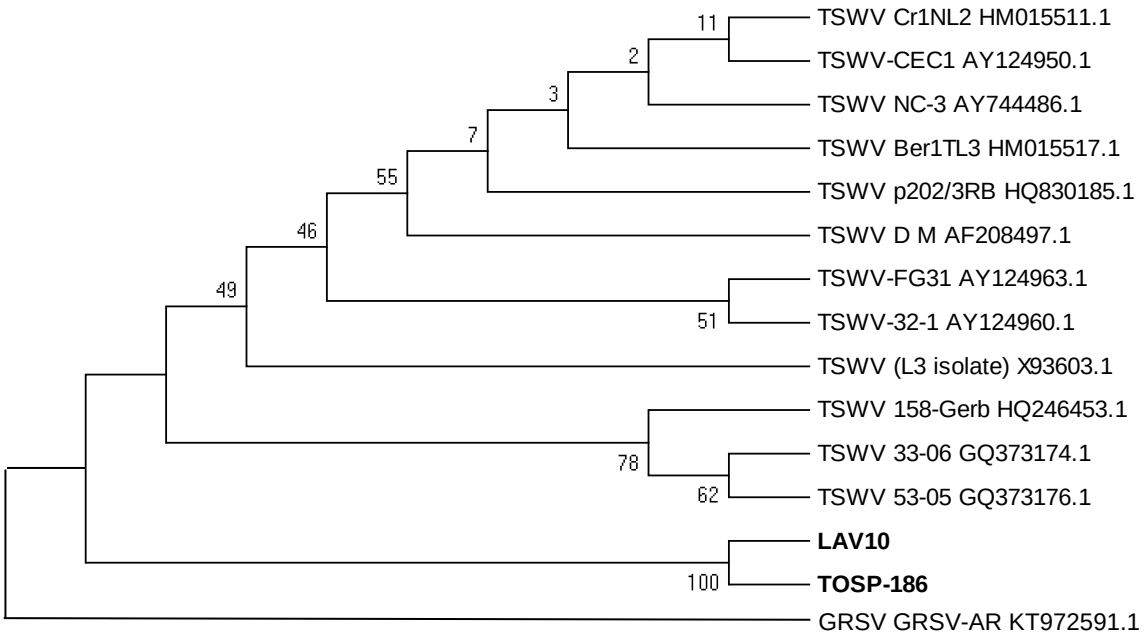

Figure 2.6: Phylogenetic relationship of the sequence of the TSWV isolates from South Africa

2.5 DISCUSSION

This is the first survey of weeds harbouring plant viruses in major tomato growing regions in South Africa. The results indicate a diverse array of plant viruses infecting various weed species.

The majority of the weeds sampled, particularly those that tested positive for viral infections, are extensively distributed throughout South Africa (Jansen van Rensburg *et al.*, 2007; Bromilow, 2010; Strathie *et al.*, 2011; SANBI, 2015). This poses a serious risk to crop production and if left unchecked may exacerbate viral incidence and distribution in agro-ecological settings. It is important to test other weeds in South Africa for viral infection particularly in areas infested with aphids, thrips and whiteflies.

The symptoms observed on both weeds and tomato crops ranged from mild to severe with mosaic patterns and chlorosis being the most prominent symptoms (Fig. 2.2). Other symptoms observed include mottling, necrotic spots, leaf curling, interveinal chlorosis and purple discoloration of leaves (Fig. 2.2). Plant viruses are exceedingly difficult to identify from symptoms alone, therefore further analysis was done to identify the virus species infecting the weeds.

Four tomato-infecting viruses were detected in four different weed species; TSWV on *A. thunbergii*, PVY on *P. peruviana*, ToCV on *S. nigrum* and a mixed infection of ToCV and ToTV on *D. stramonium* (Table 2.2; 2.4). All of these weeds, with the exception of *A. thunbergii*, are Solanaceae weeds (Table 2.2). This is characteristic of Solanaceae infecting viruses as they are known to show a predilection for Solanaceae plants. Whilst TWSV is a prominent Solanaceae infecting virus, it does have an extensive host range, affecting plant species in more than 100 plant families (Margaria *et al.*, 2015). Therefore the infection on *A. thunbergii*, whilst not being reported before to our knowledge, was probable. Interestingly similar results were found on tomato crops growing in close proximity to the weeds. These results indicate that weeds act as alternate sources of viral infection and as potential reservoirs of plant viruses. This contributes to the persistence and spread of viruses within crop fields.

Recent reports of similar proliferation of viral diseases in weed species have been documented in Belgium (Van Bogaert *et al.*, 2015), Iran (Vafaei and Mahmoodi,

2015), Kenya (Macharia *et al.*, 2016), Korea (Kwon *et al.*, 2016), Nigeria (Asala *et al.*, 2014), Saudi Arabia (Al-Shahwan *et al.*, 2016) and the United States (Srinivasan *et al.*, 2013) among many others. Many of these are first reports of weeds as hosts of a specific virus. Therefore there is a need for continuously updated information on virus identity and their distribution on weed hosts. This information is crucial for effective management of plant viral diseases.

Phylogenetic analysis is an essential component of molecular studies of plant viral diseases. The sequence of the VPg of the PVY isolate detected on *P. peruviana* in this study clustered with the previously detected South African isolate JW-186 (Moodley *et al.*, 2014) and a Zimbabwean isolate (Pepper-Zim1) reported earlier this year (Fig. 2.3; Karavina *et al.*, 2016). The high bootstrap value between these isolates suggest that they may be the same isolate or very closely related genetically (Fig. 2.3). Both the JW-186 and the Pepper-Zim1 isolates were reported on pepper plants (Moodley *et al.*, 2014; Karavina *et al.*, 2016). This suggets that it may be possible for viruses to be transmitted from weeds to crops and vice versa.

The RdRp sequence of the ToCV isolate detected on *D. stramonium* clustered with a Sudanese ToCV isolate (Sudan 3; Fig. 2.4; Fiallo-Olivé *et al.*, 2014) even though it had the closest sequence identity with the Spanish ToCV isolate (Tenerife; Table 2.3; Fiallo-Olivé *et al.*, 2014). The coat protein sequences of the ToTV isolate detected on *D. stramonium* in this study did not cluster with any of the parental ToTV isolates (Fig. 2.5).

The NSm sequence of TSWV detected on *A. thunbergii* and tomato, despite BLAST analysis showing the closest match to the Italian isolate TSWV-FG31 (Table 2.3; Finetti Sialer *et al.*, 2002), did not form a cluster with any of the parental TSWV isolates (Fig. 2.6). Interestingly, the TSWV isolate detected on *A. thunbergii* and tomato in this study were very similar even though the locations where these isolates were found were approximately 800km apart (Fig. 2.6). This further substantiates the fact that weeds are able to harbour viruses which can then subsequently be transmitted to crop plants.

The weed species that were positive for viral infections were sampled from relatively low-lying areas with warm humid climates (Eastern Cape, KwaZulu-Natal and Limpopo; Fig. 2.1). This is consistent with reports that warmer climates favour viral

disease development (Thresh *et al*., 2003). Furthermore, it has been found that warmer climates may also contribute to heightened vector populations (Broadbent, 2013). Therefore the heightened vector population observed on weeds and tomato crops may be attributed to the elevated temperatures observed in South Africa recently. This will inevitably lead to an increased incidence of viral infections on plants.

The majority of the viruses detected on the weeds were whitefly-transmitted viruses. Interestingly, even though whiteflies have been known to infest over 600 different plant species, they have shown a distinct affinity for Asteraceae, Compositae, Cruciferae, Cucurbitaceae, Euphorbiaceae, Fabaceae, Labiatae, Leguminosae, Malvaceae and Solanaceae families (Shah *et al.*, 2015). This may explain the high whitefly population observed on certain weed species**.**

Due to the scarcity of information available on non-potato PVY strains, a more comprehensive characterization of the isolate identified in this study was done. Molecular and serological analysis of the *P. peruviana*-PVY isolate indicated that there maybe a mixed infection with PVY^{C} and PVY^{O} strains (Table 2.2; 2.4; 2.5). Alternately, it is possible that a recombinant PVY^{C} strain with spliced PVY^{O}-type RNA fragments in the coat protein region may be present in the *P. peruviana* samples.

2.6 CONCLUSION

A comprehensive understanding of the ecology of plant viruses and their hosts is crucial to the development of appropriate and effective control measures. This study alludes to the potentially devastating effect weeds may have on virus incidence in South Africa, particularly given the vast density and distribution of these weeds in the country. It is of critical importance that the management of weeds be considered when developing management strategies for viral diseases on different crops.

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CHAPTER THREE

FULL GENOME ANALYSIS OF A *TOMATO SPOTTED WILT VIRUS* ISOLATE INFECTING *AMARANTHUS THUNBERGII* IN SOUTH AFRICA

3.1 ABSTRACT

Tomato spotted wilt virus (TSWV) is a genetically diverse and complex viral pathogen. It is one of the most destructive plant viruses with a considerably extensive host range. However, given its wide global distribution very few TSWV isolates have been completely sequenced. Therefore the aim of this study was to conduct a complete genome analysis of a South African TSWV isolate infecting *Amaranthus thunbergii*. The genome of TSWV was sequenced using Next-generation sequencing technology. The generated complete genome, as well as the genome organisation of the TSWV LK-1 isolate, was subjected to phylogenetic and recombination analysis. The LK-1 isolate genome organisation was found to be consistent with TSWV. Phylogenetic analysis revealed geographically diverse phylogenetic relationships of the individual open reading frames of TSWV. Recombination analysis revealed one recombination event on the S segment that showed apparent regional preference. This study provides the first complete genome sequence of a South African TSWV isolate.

3.2 INTRODUCTION

Tomato spotted wilt virus (TSWV) is one of the most biologically complex and destructive plant viral pathogens. It is currently ranked as the second most important plant virus after *Tobacco mosaic virus* (TMV; Scholthof *et al.*, 2011). TSWV is the type member of the *Tospovirus* genus, the solitary plant virus genus within the predominantly animal-infecting virus family, Bunyaviridae (Ramesh and Pappu, 2016). TSWV was first described in Australia in 1919 and later characterised as a viral pathogen in 1930 (Moyle *et al.*, 2016). Since then it has become widespread in many agroecosystems globally (Margaria and Rosa, 2015). TSWV has one of the

most extensive host ranges of any plant virus, infecting in excess of 1000 plants, including many agronomic, greenhouse and ornamental crops as well as numerous weed species (Kaye, 2011). Typical symptoms of TSWV include chlorosis, necrosis, stunting, wilting, distinctive ring spots on fruit, purple to black discolouration of foliage and leaf tip dieback (Zhang *et al.*, 2016). TSWV is exclusively transmitted by several thrips species (order Thysanoptera) in a persistent propagative manner (French *et al.*, 2016).

Like other *Tospoviruses*, TSWV consists of enveloped quasi/spherical virions and a single-stranded tripartite negative/ambisense RNA genome (Li *et al.*, 2015). The RNA fragments that comprise the TSWV genome are designated as large (L), medium (M) and small (S) segments (Margaria *et al.*, 2014). The L strand (~9 kb) has a negative polarity whilst the M (~5 kb) and S (~3 kb) strand uses ambisense replication strategy (Margaria and Rosa, 2015). The L strand codes for the replicase protein RNA dependent RNA polymerase (RdRp; Ramesh and Pappu, 2016). The positive polarity of the M strand (M(+)) encodes for a non-structural protein (NSm) responsible for cell-to-cell movement, while the negative polarity encodes for the Gn-Gc glycoproteins (Zhang *et al.*, 2016). The S(+) strand codes for a non-structural protein (NSs) associated with the suppression of gene silencing and the S(-) encodes for the nucleocapsid (N) protein (Plyusnin *et al.*, 2012).

TSWV is notoriously difficult to control due in part to its extensive host range (Sivparsad and Gubba, 2011). Recombination and reassortment of the three segments (L, M and S) results in genomic heterogeneity of TSWV, which contributes to the challenges facing control of the virus (Zhang *et al.*, 2016). The most effective control method of TSWV currently is breeding for resistance. Presently only two resistance genes have been discovered, *Sw-5* in tomato (*Solanum lycopersicum* L.) and *Tsw* in pepper (*Capsicum annuum* L.; Pappu *et al*., 2009). However, resistance-breaking strains have emerged in many parts of the world (Margaria *et al.*, 2015). Amino acid substitutions of either C to Y on codon 118 or T to N on codon 120 on the NSm ORF of the M segment is associated with the breakdown of the *Sw-5* resistance in tomato varieties (López *et al*., 2011).

Next-generation sequencing (NGS) has revolutionised plant virology in recent years. It easily facilitates the rapid acquisition of whole genome sequences of both known

and unknown viruses without having prior knowledge about the viral pathogen (Wu *et al.*, 2015). Furthermore, individual viruses can be detected from a mixed infection on a host since NGS is non-specific, thus allowing for the rapid detection of plant viruses (Kesanakurti *et al.*, 2016).

Molecular studies of the TSWV genome is a crucial step to understanding the molecular mechanism of TSWV resistance (Moyle *et al.*, 2016). This will contribute to improved alternate management strategies. Currently there are 25 complete genome sequences of TSWV isolates from Australia, Brazil, China, Italy, South Korea, Spain and the United States (Debreczeni *et al.*, 2015; Margaria and Rosa, 2015; Margaria *et al.*, 2015; Moyle *et al.*, 2016; Zhang *et al.*, 2016).

TSWV was present in South Africa as early as 1905 where it was described as a wilt of tobacco (*Nicotiana tabacum* L) plants (Thompson and van Zijl, 1996). It was later shown to be caused by the viral pathogen TSWV and was reported in the Western Cape and Free State province of South Africa in 1939 (Mathews, 1981). With the introduction of *Frankliniella occidentalis* in South Africa in 1988, the incidence of TSWV had significantly increased in the other provinces of South Africa (Thompson and van Zijl, 1996). Up until the early 2000's, TSWV was a major problem in crop cultivation in South Africa (Sivparsad and Gubba, 2011). Subsequently, there were no new cases of TSWV infection reported in South Africa. Since then TSWV was detected on tomato and *Amaranthus thunbergii* was found to be a new host of TSWV in South Africa (Chapter 2; section 2.4.2). Therefore the *A. thunbergii* isolate of TSWV was chosen for NGS to give more insight into the evolutionary biology and any recombination events that may have occurred in this TSWV isolate. From a South African perspective, very little is known about TSWV isolates detected in South Africa. No comprehensive studies of TSWV in South African have been done. Studies conducted by Sivparsad and Gubba gave limited information about the pathogenicity of TSWV (2008). Thus the aim of this study was to analyse the full genome of a South African TSWV isolate.

3.3 MATERIALS AND METHODS

3.3.1 Bioassay

Infected leaf material isolated from *A. thunbergii* was used as inoculum. The frozen leaf material was ground into a fine powder using a pestle and mortar containing liquid nitrogen. The ground leaf material was then suspended in chilled 0.1 M sodium phosphate buffer pH 7.0 with 1% sodium sulphate. Various varieties of tomato, pepper, tobacco and eggplant (*Solanum melongena* L.) were tested. Ten plants of each crop was inoculated. Mechanical inoculation was performed on leaves dusted with carborundum. Control plants were mock inoculated with sterile phosphate buffer. Experimental plants were kept at 25°C and 20°C day and night temperature in a growth room (CERU, UKZN-PMB) under 70% relative humidity, 18 hour day lengths and a light intensity of 1500 lumen per square feet. The plants were fertilised biweekly with 3 nitrogen: 1 phosphate: 3 potassium. Plants were assayed four weeks after inoculation for virus infection by symptomatology.

3.3.2 Virus isolate

A. thunbergii was found to be susceptible to TSWV during the virus survey conducted in tomato growing regions in South Africa in the 2015 and 2016 growing season. The *A. thunbergii* sample was stored at -80°C until analysed. TSWV was detected on *A. thunbergii* by performing RT-PCR using universal *Tospovirus* primers (Chapter 2, section 2.4.2). Consequently, TSWV infected leaf sample from *A. thunbergii* was randomly selected for NGS to recover the full genome of TSWV.

3.3.3 Sample preparation

Total RNA was extracted from symptomatic weed leaf tissue using the Quick-RNA™ MiniPrep Kit (Zymo Research, USA) according to manufacturers' instructions and was used as a template for NGS. RNA quality and concentration was evaluated using a NanoDrop 2000c (Thermo Scientific, USA) which was a pre-requisite for NGS on the Illumina Hiseq. Samples were pre-treated with Ribo-Zero (New England Biolabs, United Kingdom) prior to library preparation. The Ribo-Zero treatment was performed at the Agricultural Research Council's Biotechnology Platform (ARC-BTP) (Pretoria, South Africa).

3.3.4 Next-generation sequencing and data analysis

NGS was performed at ARC-BTP (Pretoria, South Africa) on the Illumina Hiseq using paired-end chemistry 125x125bp reads. Subsequent sample demultiplexing was done using the CASAVA pipeline software (Illumina, USA). The NGS generated data was analysed using the CLC Genomics Workbench v9.5.1. Read lengths less than 25 nucleotides were trimmed and pair-end sequence libraries were generated. FastQC (Andrews, 2010) was used to assess quality of the raw reads generated by NGS before and after trimming. The pair-end sequences were subsequently used as single reads in *de novo* assembly performed on the CLC Genomics Workbench according to the default parameters. Host genome data (*A. thunbergii*) was omitted from the *de novo* assembly. The contigs generated from the de *novo* assembly, were analysed by comparison with sequence data from the National Centre for Biotechnology Information (NCBI) database (www.ncbi.nml.nih.gov) using BLAST. All contigs matching to TSWV were selected for further analysis.

3.3.5 Sequence analysis and phylogeny of TSWV

All contigs that matched TSWV were selected and aligned using the Clustal W programme embedded on the Molecular Evolutionary Genetics Analysis (MEGA 6; Tamura *et al.*, 2013) software to generate the consensus genome sequence of the South African TSWV isolate. The open reading frames (ORF) on the TSWV genome were identified using ORF Finder available on the NBCI website (https://www.ncbi.nlm.nih.gov/orffinder/). The molecular weight of the proteins was determined by using the online ExPASy bioinformatics tool (Gasteiger *et al*., 2005) Phylogenetic trees of the nucleotide sequences of the five TSWV ORFs were inferred by the maximum-likelihood method based on the best evolutionary models as determined by MEGA. The Tamura 3-parameter (Gc-Gn, NSm, NSs and N; Tamura, 1992) and Tamura-Nei (RdRp; Tamura and Nei, 1993.) models were used with a Gamma distribution. The phylogenetic trees were rooted using the *Tospovirus Groundnut ring spot virus* (GRSV) as an outgroup. The bootstrap analysis was conducted using 1000 replicates. The complete genome sequences of the South African isolate of TSWV were deposited in the GenBank nucleotide database. Details of the isolates used in the phylogenetic analysis are given (Table 3.1). All alignments were performed prior to phylogenetic analysis using the Clustal W

programme on MEGA. Nucleotide and amino acid sequence compositions and sequence identities were calculated using MEGA6 and CLC workbench respectively.

Nucleotide and amino acid sequences of the NSm ORF were aligned using Clustal W on MEGA 6 to determine possible point mutations characteristic of TSWV resistance breaking isolates. The changes that are common to all resistance breaking isolates are located on the NSm amino acid sequence. C118Y or T120N substitutions on the NSm ORF is implicated in overcoming the *Sw-5* resistance in tomato varieties.

3.3.6 Recombination analysis

Recombination events of the complete genome sequence of LK-1 were identified using Recombination Detection Program 4 (RDP4) software with the default settings (Martin *et al*., 2015). The sequences of the L, M and S segment of LK-1 were used in conjunction with the isolates in Table 3.1. The analysis employed the following embedded programmes RDP (Martin and Rybicki, 2000), BootScan (Martin *et al.,* 2005), MaxChi (Smith, 1992), SiScan (Gibbs *et al*., 2000), Chimaera (Posado and Crandall, 2001), GENECONV (Padidam *et al*., 1999) and 3Seq (Boni *et al.*, 2007). Recombination events detected by at least three programmes at $p<1x10^{-6}$ were accepted.

Table 3.1: Complete genome and partial gene sequences of TSWV obtained from the NCBI nucleotide database used for phylogenetic analysis in conjunction with the complete sequences of the L, M and S of the TSWV isolate from this study.

ISOLATE	ORIGIN	GENBANK ACCESSION NO.			REFERENCES
		L	M	S	
LK-1	South Africa	KY250488	KY250489	KY250490	This study
GRSV-AR[a]	Argentina	KT972590	KT972592	KT972594	De Breuil *et al.*, 2016
D-191	Australia		HM015516		López *et al.*, 2011
WA7	Australia	KM365064			Wylie *et al.*, 2015.
Br01	Brazil	NC_002052	NC_002050	NC_002051	de Haan *et al.*, 1991
CG-1	China		JN664253	JN664252	Unpublished
KM-T	China			HQ402595	Dong *et al.*, 2010
TSWV-YN	China	JF960237	JF960236	JF960235	Hu *et al.*, 2011
p105[b]	Italy	KJ575620	KJ575621	DQ376178	Margaria *et al.*, 2015
p202/2WT[b]	Italy	KJ575619	HQ830188	HQ830187	Margaria *et al.*, 2015
-	Japan			AB088385	Unpublished
TSWV	Japan		AB010996	AB088385	Ohnishi *et al.*, 1997
wt	Japan	AB921152			Unpublished

ISOLATE	ORIGIN	GENBANK ACCESSION NO.			REFERENCES
		L	M	S	
TSWV-D	Netherlands			AF020660	Qiu *et al.*, 1998
-	South Korea	AB190813	AB190818	AB190819	Unpublished
NJ-JN	South Korea	HM581934	HM581935	HM581936	Lian *et al.*, 2013
Pep1_CY-CN	South Korea	HM581937	HM581938	HM581939	Lian *et al.*, 2013
Pep2_CY-CN	South Korea	HM58194	HM581941	HM581942	Lian *et al.*, 2013
K-4	South Korea	KC261947	KC261948	KC261949	Lian *et al.*, 2013
K-5	South Korea	KC261950	KC261951		Lian *et al.*, 2013
K-6	South Korea	KC261953	KC261954	KC261955	Lian *et al.*, 2013
K-7	South Korea	KC261956	KC261957	KC261958	Lian *et al.*, 2013
K-8	South Korea	KC261959	KC261960	KC261961	Lian *et al.*, 2013
K-10	South Korea	KC261962	KC261963	KC261964	Lian *et al.*, 2013
K-12	South Korea	KC261965	KC261966	KC261967	Lian *et al.*, 2013
K-16	South Korea	KC261968	KC261969	KC261970	Lian *et al.*, 2013
K-17	South Korea	KC261971	KC261972	KC261973	Lian *et al.*, 2013
K-18	South Korea	KC261974	KC261975	KC261976	Lian *et al.*, 2013
TSWV-MP[c]	South Africa			EF059706	Sivparsad and Gubba, 2008
TSWV-GP[c]	South Africa			EF059705	Sivparsad and Gubba, 2008
TSWV-LP[c]	South Africa			EF059704	Sivparsad and Gubba, 2008
TSWV-NW2[c]	South Africa			EF059703	Sivparsad and Gubba, 2008
TSWV-NW1[c]	South Africa			EF059702	Sivparsad and Gubba, 2008
TSWV-KZN[c]	South Africa			DQ834847	Sivparsad and Gubba, 2008
98/0472[c]	South Africa			AJ296600	Heinze *et al.*, 2001
Ab1NL2	Spain		HM015510		López *et al.*, 2011
GA-1L	Spain		FM163371		López *et al.*, 2011
GRAU	Spain		FM163370		López *et al.*, 2011
LL-N.05	Spain		FM163373		Debreczeni *et al.*, 2015
Pujol1TL3	Spain		HM015520		Debreczeni *et al.*, 2015
PVR	Spain	KP008132	KP008133	KP008134	Debreczeni *et al.*, 2015
SPAIN-1[b]	Spain		AY744492	AY744479	Tsompana *et al.*, 2005
VE427[b]	Spain			DQ376185	Unpublished
ZO	Spain		FM163372		López *et al.*, 2011
CA-1[b]	USA			AY744468	Tsompana *et al.*, 2005
CA-2[b]	USA			AY744469	Tsompana *et al.*, 2005
CA-3[b]	USA		AY744481	AY744470	Tsompana *et al.*, 2005
CA-4[b]	USA		AY744482	AY744471	Tsompana *et al.*, 2005
CA-5[b]	USA		AY744483	AY744472	Tsompana *et al.*, 2005
CA-6[b]	USA		AY744484	AY744473	Tsompana *et al.*, 2005
CA-7[b]	USA		AY744485	AY744474	Tsompana *et al.*, 2005
CO[b]	USA			AY744475	Tsompana *et al.*, 2005
D_M	USA		AF208497		Hoffmann *et al*, 2001
Hawaii	USA	AY070218			Unpublished
M[b]	USA		AY870390	AY870391	Naidu *et al.*, 2008

ISOLATE	ORIGIN	GENBANK ACCESSION NO.			REFERENCES
		L	M	S	
NC-1[b]	USA			AY744476	Tsompana *et al.*, 2005
NC-2	USA			AY744477	Tsompana *et al.*, 2005
NC-3	USA		AY744486	AY744478	Tsompana *et al.*, 2005
NC-4	USA		AY744487		Tsompana *et al.*, 2005
NC-5	USA		AY744488		Tsompana *et al.*, 2005
NC-6	USA		AY744489		Tsompana *et al.*, 2005
NC-7	USA		AY744490		Tsompana *et al.*, 2005
NC-8	USA		AY744491		Tsompana *et al.*, 2005
Regular2A	USA		AF208498		Hoffmann *et al*, 2001
T	USA		AY870389	AY870392	Unpublished
TSWV-10	USA			AF020659	Qiu *et al.*, 1998
92-tom1-zim	Zimbabwe			KU892656	Unpublished
77-pep-zim	Zimbabwe			KU884649	Unpublished
48-pep-zim	Zimbabwe			KU884648	Unpublished
Pepper-174	Zimbabwe			KU671049	Unpublished
Butternut-Harare	Zimbabwe			KT732271	Karavina *et al.*, 2016

[a] *Groundnut ring spot virus* isolate

[b] Parental strains of TSWV

[c] Partial sequences of the nucleocapsid (N) gene

3.4 RESULTS

3.4.1 Bioassay

No symptom development was observed on any of the inoculated plants. It is possible that this TSWV isolate is not easily mechanically transmissible due to some resistance in the host plants. Other inoculation techniques should be considered for future studies.

3.4.2 RNA quality assessment and Next-generation sequencing data analysis

The total RNA concentration of the sample was 1020.4ng/µl of sample and the ratio of absorbance at A_{260}/A_{280} was 2.30, which is marginally higher than the standard value of 2.0, indicating a pure RNA sample.

The size of the NGS data generated was 12.6 gigabytes which consisted of 43 675 908 raw reads (Table 3.2). A total of 33 222 contigs were generated and of these six matched to TSWV (Table 3.2). The percentage of reads mapping to TSWV

ranged from 88% to 97%. Of the six contigs generated, three contigs made up the complete genome of TSWV. Each individual contig consisted of the full sequence of the L, M and S segments. In addition to TSWV, numerous plant viruses were also detected in the *A. thunbergii* sample and these include TMV, *Potato virus Y* (PVY), *Sawbane mosaic virus*, and *Tomato leaf curl New Delhi virus* (ToLCNDV) to name a few (Data not shown).

Table 3.2: NGS assembly statistics of TSWV genome

STATISTICS	TSWV SAMPLE
No. of raw reads	43 675 908
Avg. length	125
No. of reads after trim	42 926 891
Avg. length after trim	118.8
No of reads after host removal	40 346 836
No. of contigs generated	33 222
Contigs matching to TSWV	6

3.4.3 TSWV genome organisation

The South African TSWV isolate has a genome that comprises of 16 668 nucleotides (nt). The L segment consists of 33 non-coding nucleotides at its 5' terminus followed by 8640 nucleotides coding for the RdRp protein, and 239 non-coding nucleotides at 3' terminus (Table 3.3). The ambisense M segment is made up of 100 non-coding nucleotides at its 5' proximal followed by 909 nucleotides coding for the NSm protein on the positive polarity and 3408 nucleotides on the negative polarity encoding the glycoproteins followed by 83 non-coding nucleotides at 3' proximal in succession (Table 3.3). The S segment comprises of 189 non-coding nucleotides at its 5' end followed by 1404 nucleotides coding for the NSs protein on the (+) strand and 777 on the (-) strand encoding the N protein followed by 150 non-coding nucleotides at 3' end (Table 3.3). The molecular weight of each protein is given (Table 3.3). The nucleotide and amino acid composition of LK-1 were consistent with other TSWV isolates (Table 3.4; 3.5).

Table 3.3: Genome organisation of *A. thunbergii* TSWV isolate

SEGMENT	SEGMENT LENGTH	ORF POLARITY	ORF LENGTH	POSITION OF ORF	PROTEIN CODED FOR	MW OF PROTEIN (kDa)	NO. OF AMINO ACIDS
Large	8912 nt	(-)	8640 nt	34 - 8673 nt	RdRp	331.57	2879
Medium	4820 nt	(+)	909 nt	101 - 1009 nt	NSm	33.74	302
		(-)	3408 nt	4736 -1329 nt	Gc-Gn	127.37	1135
Small	2936 nt	(+)	1404 nt	90 - 1493 nt	NSs	52.37	467
		(-)	777 nt	2785 - 2009 nt	N	28.91	258

3.4.4 Sequence and phylogenetic analysis

The nucleotide sequence identity of LK-1 compared with other TSWV isolates varied between 72 and 99% (Table 3.6). The amino acid sequence identity between the isolates also varied (Table 3.6). The Zimbabwean isolates (92-tom1-zim, 77-pep-zim, 48-pep-zim, Pepper-174. Butternut-Harare) shared the lowest nucleotide and amino acid sequence identity with LK-1, with the lowest nucleotide identity at 72.3% and the amino acid sequence identity at 0% (Table 3.6). The phylogenetic relationships of the LK-1 TSWV isolate in comparison with other global TSWV isolates differed for each ORF. The ORF of RdRp (L segment), of isolate LK-1, did not cluster with any of the parental TSWV isolates (Fig 3.1). This sequence displayed genetic diversity among other isolates and formed a distinct separate clade originating from the Brazilian (Br01) and Chinese (TSWV-YN) isolates (Fig 3.1). The Gc-Gn ORF clustered with a geographically diverse clade including the American, Australian, Brazilian, Italian, Spanish and South Korean isolates (Fig 3.2). The NSm ORF was also grouped together with a geographically diverse clade of American, Australian, Chinese, Dutch, Italian, Spanish and South Korean isolate but they were grouped in different clusters (Fig 3.3). The NSm of LK-1 formed a distinct clade with the Pujol1L3 Spanish isolate (Fig 3.3). The N ORF of LK-1 was within the same clade as other South African TSWV isolates but did not cluster together (Fig 3.4). Furthermore, in this clade, there was a range of geographically diverse isolates from America, Netherlands, Italy, and South Korea (Fig 3.4). Similarly, the NSs ORF formed a clade with the American, Italian, and South Korean isolates (Fig 3.5). The

LK-1 isolate did not have any point mutations that are associated with the *Sw-5* resistance breakdown (Fig 3.6)

Table 3.4: Nucleotide composition (%) of isolate LK-1 and other TSWV isolates.

SEGMENT	ISOLATE	T(U)	C	A	G	TOTAL
Large	LK-1	28.3	14.7	37.6	19.4	**8912.0**
	AB921152.1_wt	37.5	19.5	28.2	14.8	**8639.0**
	AY070218.1__	28.2	14.8	37.6	19.4	**8640.0**
	HM581934.1_NJ-JN	28.5	14.7	37.6	19.3	**8913.0**
	HM581937.1_Pepper1_CY-CN	28.4	14.6	37.3	19.6	**8914.0**
	HM581940.1_Pepper2_CY-CN	28.5	14.6	37.4	19.5	**8914.0**
	JF960237.1_TSWV-YN	28.5	14.5	37.7	19.3	**8910.0**
	KC261947.1_K-4	28.6	14.5	37.6	19.3	**8913.0**
	KC261950.1_K-5	28.6	14.6	37.6	19.3	**8913.0**
	KC261953.1_K-6	28.5	14.6	37.6	19.3	**8913.0**
	KC261956.1_K-7	28.5	14.6	37.6	19.3	**8913.0**
	KC261959.1_K-8	28.5	14.6	37.6	19.3	**8913.0**
	KC261962.1_K-10	28.5	14.6	37.6	19.2	**8913.0**
	KC261965.1_K-12	28.6	14.6	37.6	19.3	**8914.0**
	KC261968.1_K-16	28.5	14.6	37.6	19.3	**8913.0**
	KC261971.1_K-17	28.6	14.6	37.6	19.3	**8914.0**
	KC261974.1_K-18	28.5	14.6	37.4	19.5	**8914.0**
	KJ575619.1_p202/3WT	37.6	19.2	28.5	14.6	**8914.0**
	KJ575620.1_p105	37.5	19.5	28.4	14.6	**8912.0**
	KM365064.1_WA7	37.5	19.4	28.3	14.7	**8881.0**
	NC_002052.1_Br01	28.7	14.5	37.8	19.0	**8897.0**
	Avg.	**30.2**	**15.5**	**35.8**	**18.4**	8884.7
Medium	LK-1	32.5	17.9	31.9	17.7	**4820.0**
	AF208497.1_D_M	32.4	18.0	32.0	17.6	**4829.0**
	AF208498.1_Regular2A	32.4	17.8	32.2	17.7	**4769.0**
	AY744481.1_CA-3	32.3	17.8	32.1	17.8	**4768.0**
	AY744482.1_CA-4	32.4	17.9	31.9	17.9	**4767.0**
	AY744483.1_CA-5	32.3	17.9	31.8	17.9	**4767.0**
	AY744484.1_CA-6	32.3	18.0	31.8	17.9	**4764.0**
	AY744485.1_CA-7	32.2	18.0	32.0	17.8	**4766.0**
	AY744486.1_NC-3	32.5	17.9	32.1	17.5	**4827.0**
	AY744487.1_NC-4	32.2	18.1	31.9	17.9	**4773.0**
	AY744488.1_NC-5	32.0	18.2	32.0	17.8	**4787.0**
	AY744489.1_NC-6	32.2	18.0	31.7	18.1	**4773.0**
	AY744490.1_NC-7	32.1	18.0	31.9	17.9	**4774.0**
	AY744491.1_NC-8	32.2	18.1	32.0	17.8	**4774.0**
	AY744492.1_SPAIN-1	32.2	17.7	32.5	17.5	**4782.0**
	AY870389.1_T	32.3	18.0	31.8	18.0	**4774.0**
	AY870390.1_M	32.1	18.1	32.0	17.8	**4763.0**
	FM163372.1_ZO	32.3	18.0	31.9	17.9	**4753.0**
	FM163373.1_LL-N.05	32.3	17.9	31.8	18.0	**4752.0**
	HM015510.1_Ab1NL2	32.2	17.7	32.2	17.8	**4784.0**
	HM015516.1_D-191	32.4	18.1	32.0	17.5	**4824.0**
	HM015520.1_Pujol1TL3	32.4	18.1	32.0	17.5	**4825.0**
	HM581935.1_NJ-JN	32.3	17.7	32.4	17.6	**4783.0**
	HM581938.1_Pepper1_CY-CN	32.3	17.8	32.1	17.8	**4768.0**
	HM581941.1_Pepper2_CY-CN	32.3	17.8	32.2	17.8	**4768.0**

SEGMENT	ISOLATE	T(U)	C	A	G	TOTAL
Medium	HQ830188.1_p202/3WT	32.5	17.9	32.1	17.4	**4824.0**
	JF960236.1_TSWV-YN	32.4	17.9	32.1	17.7	**4773.0**
	JN664253.1_CG-1	32.3	17.9	32.2	17.6	**4767.0**
	KC261948.1_K-4	32.4	17.7	32.1	17.8	**4781.0**
	KC261951.1_K-5	32.3	17.7	32.4	17.6	**4792.0**
	KC261954.1_K-6	32.2	17.8	32.5	17.5	**4786.0**
	KC261957.1_K-7	32.2	17.7	32.6	17.5	**4785.0**
	KC261960.1_K-8	32.3	17.7	32.3	17.6	**4787.0**
	KC261963.1_K-10	32.3	17.7	32.3	17.7	**4791.0**
	KC261966.1_K-12	32.4	18.0	32.1	17.5	**4829.0**
	KC261969.1_K-16	32.3	17.8	32.3	17.7	**4788.0**
	KC261972.1_K-17	32.5	18.0	32.1	17.5	**4828.0**
	KC261975.1_K-18	32.3	17.7	32.2	17.8	**4770.0**
	KJ575621.1_p105	32.2	18.0	31.8	17.9	**4766.0**
	NC_002050.1_Br01	32.5	18.0	31.8	17.6	**4821.0**
	Avg.	**32.3**	**17.9**	**32.1**	**17.7**	**4784.8**
Small	LK-1	33.7	18.7	31.4	16.2	**2936.0**
	NC_002051.1_Br01	33.0	19.3	31.6	16.0	**2916.0**
	KC261976.1_K-18	34.0	18.2	32.5	15.3	**3020.0**
	KC261973.1_K-17	33.7	18.5	31.6	16.2	**2961.0**
	KC261970.1_K-16	33.8	18.4	32.1	15.8	**2973.0**
	KC261967.1_K-12	33.7	18.5	31.6	16.1	**2961.0**
	KC261964.1_K-10	33.9	18.4	31.9	15.8	**2975.0**
	KC261961.1_K-8	33.9	18.3	32.0	15.8	**2977.0**
	KC261958.1_K-7	33.6	18.5	32.1	15.8	**2967.0**
	KC261955.1_K-6	33.6	18.5	32.0	15.9	**2969.0**
	KC261949.1_K-4	33.8	18.4	32.0	15.8	**2971.0**
	JN664252.1_CG-1	33.6	18.8	31.7	15.9	**2920.0**
	JF960235.1_TSWV-YN	34.0	18.5	31.9	15.7	**2970.0**
	HQ830187.1_p202/3WT	33.9	18.5	31.6	16.1	**2963.0**
	HQ402595.1_KM-T	33.9	18.5	31.9	15.7	**2971.0**
	HM581942.1_Pepper2_CY-CN	34.1	18.2	32.4	15.3	**3013.0**
	HM581939.1_Pepper1_CY-CN	34.1	18.2	32.4	15.3	**3013.0**
	HM581936.1_NJ-JN	33.6	18.5	32.1	15.8	**2968.0**
	DQ376185.1_VE427	33.4	18.8	31.7	16.2	**2922.0**
	DQ376178.1_P105	33.6	18.7	31.6	16.0	**2927.0**
	AY870392.1_T	33.8	18.7	31.4	16.1	**3016.0**
	AY870391.1_M	35.0	18.1	30.3	16.6	**3047.0**
	AY744479.1_SPAIN-1	33.3	18.9	31.6	16.3	**2922.0**
	AY744478.1_NC-3	33.6	18.6	31.7	16.1	**2954.0**
	AY744477.1_NC-2	34.2	18.5	31.3	16.1	**3021.0**
	AY744476.1_NC-1	33.5	18.7	31.6	16.2	**2959.0**
	AY744475.1_CO	33.4	18.8	31.6	16.1	**2923.0**
	AY744474.1_CA-7	33.3	18.9	31.9	15.9	**2927.0**
	AY744473.1_CA-6	33.4	18.8	31.7	16.1	**2920.0**
	AY744472.1_CA-5	33.3	18.9	31.7	16.1	**2921.0**
	AY744471.1_CA-4	33.4	18.9	31.7	16.0	**2921.0**
	AY744470.1_CA-3	33.4	18.8	31.7	16.0	**2921.0**
	AY744469.1_CA-2	33.3	18.9	31.9	16.0	**2926.0**
	AY744468.1_CA-1	33.3	18.9	31.8	16.0	**2927.0**
	AF020660.1_TSWV-D	33.7	18.5	31.7	16.1	**2955.0**
	AF020659.1_TSWV-10	34.1	18.5	31.4	16.0	**3017.0**
	Avg.	**33.7**	**18.6**	**31.7**	**15.9**	**2961.3**

Table 3.5: Amino acid composition (%) of isolate LK-1 and other TSWV isolates.

Isolates	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
Large segment																					
LK-1	3.3	3.6	3.7	5.4	5.2	3.4	2.6	7.6	10.4	9.4	2.7	5.7	2.5	4.1	7.4	7.1	5.7	4.7	1.5	3.8	**2680**
wt	3.1	4.1	2.2	2.5	10.5	2.4	3.6	9.1	5.2	13.6	2.0	4.4	3.8	3.2	3.7	12.2	4.5	5.2	1.1	3.6	**2639**
__	3.4	3.5	3.6	5.5	5.2	3.7	2.6	7.8	10.6	9.3	2.7	5.8	2.5	4.0	7.1	7.0	5.8	4.5	1.6	3.8	**2599**
NJ-JN	3.1	3.5	3.5	5.3	5.4	3.7	2.7	7.8	10.4	9.6	2.7	6.0	2.4	4.3	7.0	7.0	5.7	4.9	1.4	3.8	**2679**
Pepper1_CY-CN	3.2	3.4	3.7	5.7	5.4	4.0	2.5	7.4	10.0	9.3	2.6	5.6	2.3	4.0	7.5	7.3	6.0	4.7	1.4	3.9	**2681**
Pepper2_CY-CN	3.1	3.4	3.8	5.6	5.4	4.0	2.5	7.4	10.1	9.2	2.6	5.6	2.3	4.0	7.4	7.3	6.0	4.8	1.4	3.9	**2681**
TSWV-YN	3.2	3.4	3.9	5.3	5.4	3.6	2.5	7.8	10.3	9.5	2.7	5.7	2.1	4.2	7.5	7.3	5.6	4.6	1.5	3.9	**2685**
K-4	3.0	3.4	3.5	5.4	5.4	3.7	2.7	7.7	10.5	9.7	2.7	6.0	2.3	4.1	7.0	7.0	5.7	5.0	1.5	3.8	**2679**
K-5	3.1	3.5	3.5	5.3	5.4	3.8	2.7	7.8	10.4	9.7	2.7	6.0	2.4	4.1	6.9	7.0	5.7	4.8	1.5	3.9	**2675**
K-6	3.1	3.5	3.5	5.3	5.4	3.7	2.7	7.7	10.5	9.6	2.7	6.0	2.3	4.2	7.0	7.0	5.7	4.9	1.4	3.8	**2679**
K-7	3.1	3.5	3.5	5.4	5.4	3.7	2.7	7.7	10.4	9.6	2.7	5.9	2.4	4.2	6.9	7.0	5.8	4.9	1.4	3.8	**2678**
K-8	3.1	3.4	3.5	5.3	5.4	3.7	2.7	7.7	10.5	9.6	2.7	6.0	2.4	4.1	6.9	6.9	5.8	4.9	1.5	3.8	**2677**
K-10	3.1	3.5	3.5	5.3	5.4	3.7	2.7	7.7	10.4	9.6	2.7	6.0	2.4	4.1	7.0	7.1	5.8	4.9	1.5	3.8	**2677**
K-12	3.2	3.5	3.4	5.3	5.3	3.8	2.6	7.6	10.3	9.5	2.7	6.0	2.4	4.2	7.1	7.0	5.8	4.7	1.5	3.9	**2679**
K-16	3.1	3.5	3.5	5.3	5.4	3.7	2.7	7.6	10.4	9.6	2.7	6.0	2.4	4.1	7.1	6.9	5.8	5.0	1.5	3.8	**2677**
K-17	3.2	3.6	3.5	5.4	5.4	3.7	2.6	7.6	10.3	9.4	2.7	6.1	2.4	4.2	7.1	7.0	5.8	4.7	1.5	3.9	**2680**
K-18	3.1	3.4	3.8	5.6	5.4	4.0	2.5	7.5	10.1	9.2	2.7	5.6	2.3	4.0	7.3	7.4	6.0	4.7	1.4	3.9	**2678**
p202/3WT	3.3	4.2	2.0	2.6	10.7	2.3	3.7	9.5	4.9	13.6	1.8	4.6	3.7	3.4	3.7	11.8	4.4	5.1	0.9	3.7	**2722**
p105	3.1	4.0	2.2	2.5	10.3	2.3	3.6	9.4	5.2	13.9	1.8	4.6	3.5	3.1	3.8	12.4	4.8	5.1	1.0	3.6	**2721**
WA7	3.4	4.1	2.0	2.6	10.6	2.5	3.6	9.3	5.0	13.5	1.9	4.5	3.8	3.4	3.7	12.0	4.4	5.1	0.8	3.8	**2711**
Br01	3.2	3.5	3.5	5.3	5.4	3.5	2.6	7.8	10.4	9.4	2.7	5.9	2.4	4.2	7.3	7.7	5.4	4.7	1.3	4.0	**2664**
Avg.	**3.2**	**3.6**	**3.3**	**4.8**	**6.4**	**3.5**	**2.8**	**8.0**	**9.3**	**10.3**	**2.5**	**5.6**	**2.6**	**3.9**	**6.5**	**8.1**	**5.5**	**4.9**	**1.4**	**3.8**	2678
Medium segment																					
LK-1	4.1	2.1	3.4	5.0	6.3	4.5	2.1	8.5	7.6	10.8	2.5	4.3	5.1	3.5	3.0	11.9	5.3	6.7	1.0	2.3	**1544**
__	4.1	2.4	3.4	4.9	5.9	4.4	2.0	8.6	7.6	10.9	3.0	4.4	5.3	3.4	3.2	11.9	4.9	6.7	0.9	2.3	**1520**
D_M	4.0	2.1	3.4	4.6	5.9	4.6	2.1	8.7	8.3	10.9	2.4	4.3	5.0	3.4	3.0	12.2	5.2	6.7	1.0	2.4	**1549**
Regular2A	4.1	2.2	3.2	4.9	5.8	4.3	2.1	8.6	7.7	11.2	3.0	4.5	5.1	3.3	3.3	11.9	4.9	6.8	0.9	2.4	**1524**
CA-3	4.1	2.3	3.4	4.7	5.8	4.4	2.0	8.3	7.7	10.9	2.9	4.3	5.2	3.4	3.4	12.0	5.2	6.9	0.9	2.3	**1523**
CA-4	4.1	2.2	3.3	4.9	5.7	4.4	2.0	8.5	7.6	11.0	2.9	4.3	5.2	3.4	3.3	12.0	5.0	6.9	0.9	2.4	**1524**
CA-5	4.1	2.2	3.3	4.9	5.6	4.4	2.0	8.5	7.7	11.0	2.8	4.3	5.2	3.4	3.3	12.1	5.1	6.8	0.9	2.4	**1524**
CA-6	4.1	2.2	3.3	4.9	5.6	4.4	2.0	8.5	7.6	11.0	2.8	4.3	5.2	3.4	3.3	12.1	5.1	6.8	0.9	2.4	**1524**
CA-7	4.1	2.3	3.3	4.7	5.8	4.5	2.0	8.4	7.7	11.0	2.9	4.3	5.2	3.4	3.2	12.0	5.1	6.8	0.9	2.3	**1523**
NC-3	4.1	2.1	3.4	4.8	6.0	4.5	2.1	8.7	8.1	10.9	2.4	4.3	5.2	3.4	3.1	11.9	5.2	6.6	1.0	2.4	**1546**
NC-4	4.2	2.2	3.3	4.9	5.8	4.4	2.0	8.3	7.5	10.9	2.9	4.3	5.1	3.5	3.3	12.0	5.2	6.7	1.0	2.4	**1528**
NC-5	4.1	2.2	3.3	4.9	5.6	4.5	2.0	8.2	7.5	11.0	2.9	4.4	5.2	3.4	3.3	11.9	5.2	6.8	1.0	2.5	**1532**

Isolates	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
Medium segment																					
NC-6	4.2	2.3	3.3	4.9	5.8	4.4	2.0	8.4	7.4	11.0	2.9	4.3	5.1	3.3	3.5	12.0	5.1	6.7	1.0	2.4	**1527**
NC-7	4.2	2.4	3.2	5.0	5.7	4.4	2.0	8.4	7.5	11.0	2.9	4.4	5.1	3.4	3.3	12.0	5.0	6.7	1.0	2.4	**1528**
NC-8	4.1	2.3	3.3	5.0	5.7	4.3	2.0	8.4	7.5	11.1	2.8	4.4	5.1	3.4	3.3	12.0	5.2	6.7	1.0	2.5	**1529**
SPAIN-1	4.0	2.2	3.3	4.9	5.7	4.3	2.0	8.4	7.8	11.1	2.8	4.6	5.1	3.5	3.1	11.6	5.2	6.9	0.9	2.4	**1530**
T	4.3	2.2	3.2	4.9	5.7	4.4	2.2	8.3	7.6	11.1	2.9	4.3	5.2	3.3	3.3	11.9	5.1	6.7	1.0	2.4	**1529**
M	4.3	2.2	3.3	4.9	5.8	4.3	2.0	8.3	7.5	11.2	2.8	4.3	5.3	3.5	3.2	11.7	5.3	6.8	1.1	2.4	**1523**
ZO	4.3	2.0	3.4	4.9	5.9	4.5	2.0	8.5	7.7	11.2	2.9	4.3	5.0	3.3	3.2	12.0	4.9	6.8	1.1	2.4	**1520**
LL-N.05	4.2	2.1	3.4	4.9	5.8	4.5	2.0	8.6	7.4	11.1	2.9	4.3	5.1	3.3	3.2	12.0	4.8	6.8	1.1	2.4	**1519**
Ab1NL2	4.1	2.2	3.3	5.0	5.6	4.5	2.0	8.4	7.7	11.1	2.8	4.5	5.2	3.5	3.0	11.9	5.0	6.9	0.9	2.4	**1528**
D-191	4.0	1.9	3.5	5.0	6.1	4.4	2.1	8.6	7.6	10.8	2.5	4.2	5.2	3.4	3.0	12.1	5.3	6.6	0.9	2.7	**1544**
Pujol1TL3	4.2	2.0	3.4	4.8	6.1	4.4	2.1	8.5	7.8	10.9	2.5	4.2	5.1	3.4	3.2	12.1	5.1	6.6	1.0	2.5	**1546**
NJ-JN	4.1	2.2	3.3	4.8	5.7	4.4	2.0	8.4	7.8	11.1	2.8	4.6	5.1	3.5	3.1	12.0	4.9	6.9	0.9	2.4	**1527**
Pepper1_CY-CN	4.1	2.4	3.4	4.8	5.6	4.3	2.0	8.5	8.0	11.1	2.7	4.1	5.1	3.5	3.1	12.2	5.1	6.8	0.9	2.3	**1524**
Pepper2_CY-CN	4.1	2.4	3.4	4.9	5.6	4.3	2.0	8.5	7.9	11.1	2.7	4.1	5.2	3.5	3.1	12.1	5.1	6.8	0.9	2.3	**1523**
p202/3WT	4.1	2.1	3.4	4.8	6.1	4.4	2.1	8.7	8.0	10.9	2.4	4.2	5.2	3.4	3.0	12.1	5.1	6.7	1.0	2.3	**1544**
TSWV-YN	4.1	2.3	3.3	4.9	5.6	4.5	2.0	8.4	7.6	11.3	3.0	4.7	5.2	3.4	3.1	11.7	5.0	6.7	0.8	2.6	**1525**
CG-1	4.1	2.3	3.4	4.8	5.7	4.4	2.0	8.4	7.8	11.2	3.0	4.5	5.1	3.4	3.1	11.9	5.1	6.6	0.7	2.5	**1521**
K-4	4.0	2.2	3.3	4.8	5.6	4.5	2.0	8.2	7.7	11.1	2.9	4.5	5.2	3.5	3.0	11.9	5.0	6.9	0.9	2.5	**1528**
K-5	4.2	2.2	3.4	4.9	5.7	4.4	2.0	8.3	7.9	11.4	2.8	4.4	5.0	3.5	3.0	11.7	5.0	6.7	0.9	2.4	**1533**
K-6	4.2	2.2	3.3	4.9	5.7	4.4	2.1	8.4	7.8	11.1	2.8	4.6	5.1	3.5	3.0	11.8	4.9	6.8	0.9	2.4	**1530**
K-7	4.1	2.2	3.3	4.9	5.7	4.3	2.0	8.4	7.8	11.1	2.8	4.6	5.1	3.5	3.1	11.8	4.9	6.8	0.9	2.4	**1529**
K-8	4.2	2.2	3.4	4.9	5.6	4.4	2.0	8.3	7.8	11.2	2.7	4.5	5.1	3.5	2.9	11.8	5.0	6.8	0.9	2.4	**1529**
K-10	4.2	2.2	3.4	4.8	5.7	4.4	2.0	8.3	8.0	11.3	2.8	4.4	5.1	3.5	3.0	11.8	5.0	6.8	0.9	2.4	**1530**
K-12	4.1	2.1	3.5	4.8	5.9	4.5	2.1	8.7	8.0	10.8	2.4	4.4	5.2	3.4	3.1	12.1	5.1	6.6	1.0	2.4	**1550**
K-16	4.2	2.2	3.4	4.9	5.6	4.4	2.0	8.2	7.9	11.2	2.9	4.4	5.1	3.5	2.9	11.8	5.0	6.9	0.9	2.4	**1530**
K-17	4.1	2.1	3.5	4.8	5.9	4.5	2.1	8.8	8.1	10.9	2.4	4.4	5.2	3.4	3.0	12.1	5.0	6.6	1.0	2.4	**1550**
K-18	4.1	2.3	3.3	4.9	5.6	4.3	2.0	8.5	7.8	11.1	2.7	4.2	5.1	3.5	3.2	12.0	5.2	6.8	0.9	2.4	**1525**
p105	4.1	2.3	3.3	4.9	5.9	4.4	2.0	8.4	7.4	11.1	2.8	4.4	5.1	3.4	3.2	11.9	5.1	7.0	0.9	2.4	**1523**
Br01	4.1	2.0	3.4	4.9	6.6	4.5	2.1	8.6	7.7	10.7	2.5	4.2	5.1	3.2	3.4	12.0	5.1	6.5	0.9	2.4	**1541**
Avg.	**4.1**	**2.2**	**3.4**	**4.9**	**5.8**	**4.4**	**2.0**	**8.4**	**7.7**	**11.0**	**2.8**	**4.4**	**5.1**	**3.4**	**3.2**	**11.9**	**5.1**	**6.8**	**0.9**	**2.4**	1530
Small segment																					
LK-1	3.3	4.5	2.3	2.4	7.8	2.9	2.6	7.0	8.4	15.8	2.1	3.2	3.9	6.5	3.8	10.7	5.3	3.8	1.0	2.8	**906**
Br01	3.8	4.2	2.5	2.6	7.5	2.2	2.6	6.5	7.8	14.8	2.1	3.8	3.9	6.1	4.7	11.0	6.3	3.7	0.9	3.0	**896**
K-18	3.4	3.8	2.5	2.8	8.8	2.0	2.7	7.2	9.3	15.5	2.0	3.5	2.9	6.7	3.6	9.9	5.8	3.3	1.1	3.2	**938**
K-17	3.3	4.2	2.5	2.7	8.0	2.7	2.5	7.0	8.2	15.9	2.0	3.5	3.3	6.7	3.7	10.6	5.8	3.6	1.1	2.7	**914**
K-16	3.3	4.1	2.7	2.6	8.2	2.4	2.5	7.1	8.4	15.5	2.0	3.6	3.4	6.6	3.8	10.0	5.9	3.4	1.2	3.3	**916**
K-12	3.1	4.2	2.5	2.6	7.8	2.7	2.6	7.0	8.4	16.0	2.0	3.4	3.3	6.6	3.7	10.8	5.7	3.8	1.1	2.8	**915**

Isolates	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
Small segment																					
K-10	3.3	4.2	2.7	2.5	8.3	2.4	2.5	7.2	8.5	15.4	2.0	3.5	3.4	6.7	3.8	10.1	5.8	3.4	1.1	3.3	**915**
K-8	3.3	4.1	2.7	2.6	8.4	2.4	2.5	7.4	8.5	15.4	2.0	3.5	3.4	6.5	3.9	10.0	5.7	3.4	1.1	3.3	**918**
K-7	3.2	4.2	2.6	2.5	8.2	2.5	2.5	6.8	8.6	15.5	2.0	3.5	3.4	6.7	3.8	10.1	6.1	3.5	1.1	3.2	**912**
K-6	3.2	4.2	2.6	2.6	8.2	2.5	2.5	6.9	8.5	15.4	2.0	3.5	3.4	6.7	3.8	10.1	6.0	3.5	1.1	3.3	**914**
K-4	3.4	4.2	2.6	2.7	8.3	2.4	2.6	7.2	8.4	15.2	2.1	3.5	3.5	6.6	3.6	10.4	5.6	3.4	1.1	3.3	**915**
CG-1	3.2	4.0	2.7	2.9	7.9	2.1	2.7	6.9	7.9	15.7	2.2	3.3	3.6	6.2	4.0	10.4	6.4	3.8	1.0	3.0	**897**
TSWV-YN	3.1	4.1	2.5	2.9	8.2	2.4	2.7	7.2	8.2	16.0	2.0	3.4	3.1	6.9	3.5	10.4	6.0	3.5	1.0	2.9	**912**
p202/3WT	3.2	4.2	2.5	2.6	8.1	2.7	2.5	6.9	8.3	16.0	2.2	3.3	3.3	6.6	3.7	10.5	5.6	3.7	1.1	3.0	**913**
KM-T	3.1	4.1	2.6	2.8	8.1	2.2	2.8	7.0	8.2	16.0	2.1	3.4	3.2	6.8	3.7	10.4	6.0	3.6	1.0	2.8	**913**
Pepper2_CY-CN	3.4	4.0	2.5	2.8	9.1	2.0	2.7	7.0	9.1	15.4	2.0	3.5	3.0	6.7	3.6	9.8	5.8	3.4	1.0	3.2	**935**
Pepper1_CY-CN	3.4	4.0	2.5	2.8	9.1	2.0	2.6	7.1	9.1	15.4	2.0	3.4	3.0	6.7	3.6	9.8	5.8	3.4	1.0	3.3	**935**
NJ-JN	3.2	4.2	2.6	2.6	8.2	2.5	2.5	6.9	8.5	15.4	2.0	3.5	3.4	6.7	3.8	10.1	6.0	3.5	1.1	3.3	**914**
VE427	3.6	4.3	2.6	2.3	7.8	2.7	2.7	6.4	8.6	14.9	1.9	3.3	3.4	6.8	3.4	11.4	5.7	3.8	1.0	3.4	**900**
P105	3.4	4.3	2.4	2.3	7.6	2.8	2.9	6.8	8.4	15.1	2.2	3.8	3.7	6.5	3.7	10.9	5.3	3.9	1.0	3.0	**902**
T	3.8	4.3	2.3	2.4	8.0	2.6	2.8	6.7	8.3	15.4	2.0	3.6	3.7	6.5	3.7	11.0	5.6	3.7	1.0	3.0	**929**
M	3.5	5.6	2.3	2.2	8.8	2.5	2.4	6.2	7.7	15.5	2.0	3.6	3.2	6.0	3.9	10.3	5.5	4.5	1.0	3.2	**943**
SPAIN-1	3.7	4.3	2.6	2.3	7.9	2.8	2.7	6.3	8.4	14.9	2.0	3.3	3.8	6.8	3.4	11.0	5.8	3.7	1.0	3.3	**900**
NC-3	3.2	4.2	2.5	2.6	7.7	2.8	2.6	6.9	8.4	15.7	2.0	3.4	3.4	6.6	3.7	10.8	5.7	3.7	1.1	2.9	**909**
NC-2	3.8	4.5	2.4	2.3	8.1	2.6	2.7	6.5	8.0	15.6	2.0	3.6	3.6	6.8	3.3	10.8	5.7	3.8	1.0	2.9	**933**
NC-1	3.2	4.2	2.4	2.7	7.6	2.8	2.6	6.9	8.3	15.9	2.0	3.5	3.4	6.6	3.7	10.8	5.7	3.7	1.1	2.8	**913**
CO	3.4	4.2	2.4	2.4	8.0	2.7	2.7	6.5	8.1	14.7	2.1	3.6	3.8	6.7	3.8	10.9	5.7	4.1	1.0	3.2	**899**
CA-7	3.7	4.3	2.4	2.4	7.7	2.4	2.7	6.8	8.7	15.0	2.1	3.4	3.7	6.9	3.6	11.0	5.7	3.6	0.9	3.0	**899**
CA-6	3.6	4.4	2.6	2.2	7.5	2.6	2.7	6.8	8.5	15.1	2.2	3.5	3.7	6.9	3.7	11.2	5.5	3.7	0.9	2.9	**895**
CA-5	3.6	4.5	2.6	2.2	7.5	2.6	2.7	6.8	8.5	15.1	2.2	3.5	3.8	6.9	3.7	11.2	5.5	3.7	0.9	2.8	**896**
CA-4	3.6	4.5	2.6	2.2	7.5	2.6	2.8	6.8	8.4	15.2	2.2	3.5	3.7	7.0	3.6	11.2	5.5	3.7	0.9	2.8	**896**
CA-3	3.6	4.6	2.5	2.3	7.6	2.5	2.7	6.8	8.5	15.1	2.2	3.6	3.7	6.9	3.8	11.2	5.5	3.7	0.8	2.7	**896**
CA-2	3.6	4.3	2.4	2.3	7.6	2.6	2.7	6.9	8.6	14.9	2.1	3.5	3.7	6.9	3.7	11.1	5.6	3.7	0.9	3.0	**898**
CA-1	3.6	4.3	2.4	2.3	7.6	2.6	2.7	6.9	8.6	14.9	2.1	3.4	3.9	6.9	3.7	11.0	5.6	3.7	0.9	3.0	**899**
TSWV-D	3.2	4.2	2.5	2.5	7.8	2.6	2.5	6.8	8.5	15.8	2.0	3.3	3.3	6.5	4.1	10.9	5.7	3.8	1.1	2.9	**910**
TSWV-10	3.7	4.2	2.4	2.5	8.0	2.5	2.8	7.0	8.1	15.8	2.0	3.4	3.4	6.9	3.2	11.2	5.3	3.7	1.1	3.0	**930**
__	3.4	4.1	2.2	2.8	8.7	2.6	2.5	6.8	8.6	16.1	2.0	3.1	3.7	6.3	3.5	10.4	5.9	3.5	0.9	3.1	**922**
Avg.	**3.4**	**4.3**	**2.5**	**2.5**	**8.0**	**2.5**	**2.6**	**6.9**	**8.4**	**15.4**	**2.1**	**3.5**	**3.5**	**6.7**	**3.7**	**10.6**	**5.7**	**3.7**	**1.0**	**3.0**	**912**

Table 3.6: Percentage nucleotide and protein sequence identity between LK-1 and other TSWV isolates

ISOLATE	RdRp		Gc-Gn		NSm		N		NSs	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
D-191	-	-	95.3	96.2	98.1	100	-	-	-	-
WA7	98.1	100	-	-	-	-	-	-	-	-
Br01	95.2	93.3	94.3	92.1	93.3	93.6	98.2	93.6	95.3	100
CG-1	-	-	97.3	92.1	97.7	93.6	96.3	100	96.1	100
KM-T	-	-	-	-	-	-	98.6	93.2	96.3	100
TSWV-YN	96.2	93.2	98.6	93.2	95.3	96.2	92.3	93.6	92.2	93.3
p105	96.2	92.1	98.7	100	98.2	96.2	94.3	92.1	98.1	100
p202/2WT	97.3	93.2	93.2	100	95.3	96.2	95.1	100	95.2	100
-	-	-	-	-	-	-	94.6	94.2	95.4	93.3
TSWV	-	-	95.3	96.2	98.1	100	97.3	100	96.3	93.3
wt	96.3	96.2	-	-	-	-	-	-	-	-
TSWV-D	-	-	-	-	-	-	96.1	93.6	98.6	93.2
-	98.1	93.5	95.7	96.2	92.2	93.3	97.3	93.2	98.7	100
NJ-JN	95.2	91.4	95.6	96.4	98.1	100	97.3	92.1	93.2	100
Pep1_CY-CN	95.4	91.6	94.7	96.5	95.2	100	98.2	93.2	95.3	96.2
Pep2_CY-CN	96.3	91.8	95.2	100	95.4	93.3	97.6	93.4	98.2	96.2
K-4	92.2	91.3	95.1	100	96.3	93.3	95.3	100	96.2	93.3
K-5	98.1	92.1	95.1	96.3	92.2	93.3	-	-	-	-
K-6	95.2	92.5	94.7	92.4	98.1	100	97.5	100	98.2	100
K-7	98.1	93.5	97.1	93.4	95.2	100	95.3	100	97.2	100
K-8	98.1	91.4	95.7	96.2	95.4	93.3	96.2	93.4	96.2	100
K-10	95.2	91.6	95.6	96.4	96.3	93.3	97.6	93.2	96.2	100
K-12	95.4	91.8	94.7	96.5	96.2	100	96.3	92.1	97.3	100
K-16	96.3	91.3	95.2	100	97.3	100	98.3	92.5	98.2	96.3
K-17	92.2	92.1	95.1	100	98.2	96.3	97.2	93.4	95.3	96.2
K-18	98.1	92.5	95.1	96.3	95.3	96.2	94.3	100	93.2	100
TSWV-MP	-	-	-	-	-	-	95.4	91.4	-	-
TSWV-GP	-	-	-	-	-	-	93.3	91.6	-	-
TSWV-LP	-	-	-	-	-	-	97.9	91.8	-	-
TSWV-NW2	-	-	-	-	-	-	92.3	91.3	-	-
TSWV-NW1	-	-	-	-	-	-	91.1	92.1	-	-
TSWV-KZN	-	-	-	-	-	-	94.2	92.5	-	-
98/0472	-	-	-	-	-	-	91.2	91.1	-	-
Ab1NL2	-	-	98.3	93.5	97.3	93.2	-	-	-	-
GA-1L	-	-	95.4	91.4	97.3	92.1	-	-	-	-
GRAU	-	-	93.3	91.6	98.2	93.2	-	-	-	-
LL-N.05	-	-	97.9	91.8	97.6	93.4	-	-	-	-
Pujol1TL3	-	-	92.3	91.3	95.3	94.6	-	-	-	-
PVR	92.4	91.3	91.1	92.1	97.3	93.2	98.6	100	98.3	100
SPAIN-1	-	-	94.2	92.5	97.3	92.1	97.2	100	97.1	100

ISOLATE	RdRp		Gc-Gn		NSm		N		NSs	
	nt	aa	nt	aa	nt		nt	aa	nt	aa
VE427	-	-	-	-	-	-	97.3	100	94.8	100
ZO	-	-	92.1	98.1	97.9	91.8	-	-	-	-
CA-1	-	-	-	-	-	-	94.3	100	95.7	96.2
CA-2	-	-	-	-	-	-	94.6	100	95.6	96.4
CA-3	-	-	95.4	91.4	97.2	100	98.2	95.6	94.7	96.5
CA-4	-	-	93.3	91.6	96.2	100	98.3	93.5	95.2	100
CA-5	-	-	97.9	91.8	96.2	100	92.3	93.6	95.1	100
CA-6	-	-	92.3	91.3	97.3	100	91.3	91.5	95.1	96.3
CA-7	-	-	91.1	92.1	98.2	96.3	97.4	91.2	94.7	92.4
CO	-	-	-	-	-	-	97.3	100	97.1	93.4
D_M	-	-	92.4	93.6	95.6	96.4	-	-	-	-
Hawaii	93.6	91.3	-	-	-	-	-	-	-	-
M	-	-	95.1	96.3	95.6	94.7	98.3	92.5	98.3	92.4
NC-1	-	-	-	-	-	-	95.6	93.6	93.3	93.6
NC-2	-	-	-	-	-	-	94.9	94.1	97.7	93.6
NC-3	-	-	98.1	100	98.3	93.5	98.1	91.2	92.2	94.1
NC-4	-	-	95.2	100	98.3	93.5	-	-	-	-
NC-5	-	-	95.4	93.3	95.4	91.4	-	-	-	-
NC-6	-	-	96.3	93.3	93.3	91.6	-	-	-	-
NC-7	-	-	92.2	93.3	97.9	91.8	-	-	-	-
NC-8	-	-	98.1	100	92.3	91.3	-	-	-	-
Regular2A	-	-	95.2	100	91.1	92.1	-	-	-	-
T	-	-	98.1	100	94.2	92.5	91.3	95.2	98.1	100
TSWV-10	-	-	-	-	-	-	97.5	91.1	94.2	100
92-tom1-zim	-	-	-	-	-	-	72.3	26.7	-	-
77-pep-zim	-	-	-	-	-	-	74.3	0	-	-
48-pep-zim	-	-	-	-	-	-	78.1	42.9	-	-
Pepper-174	-	-	-	-	-	-	74.6	26.9	-	-
Butternut-Harare	-	-	-	-	-	-	76.1	26.7	-	-

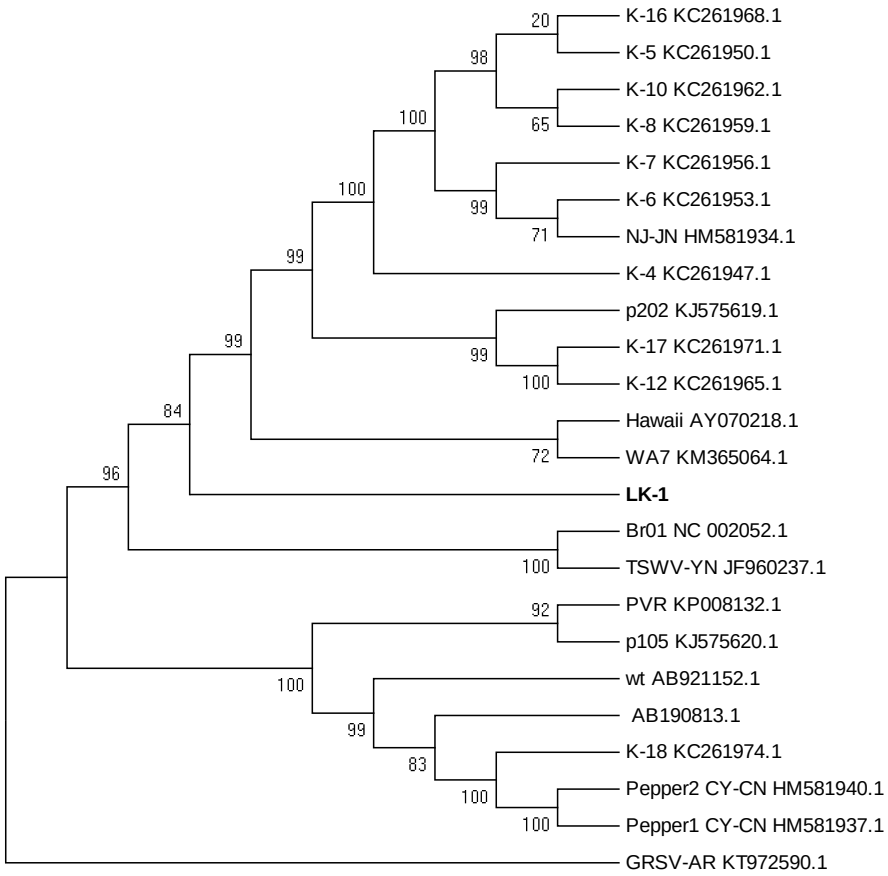


Figure 3.1: Phylogenetic relationship of the RdRp sequences of the LK-1 TSWV isolate

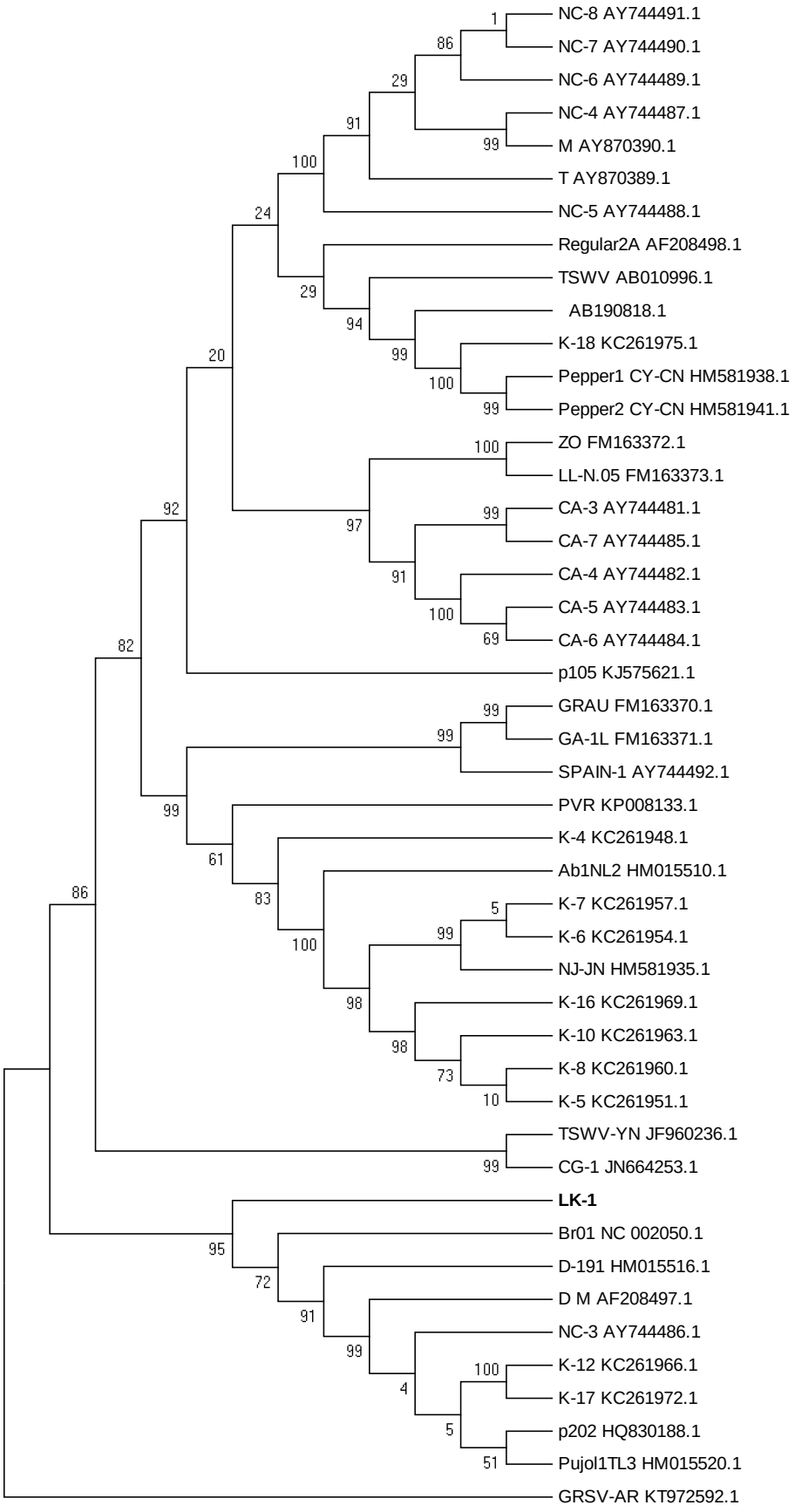


Figure 3.2: Phylogenetic relationship of the Gc-Gn sequences of the LK-1 TSWV isolate

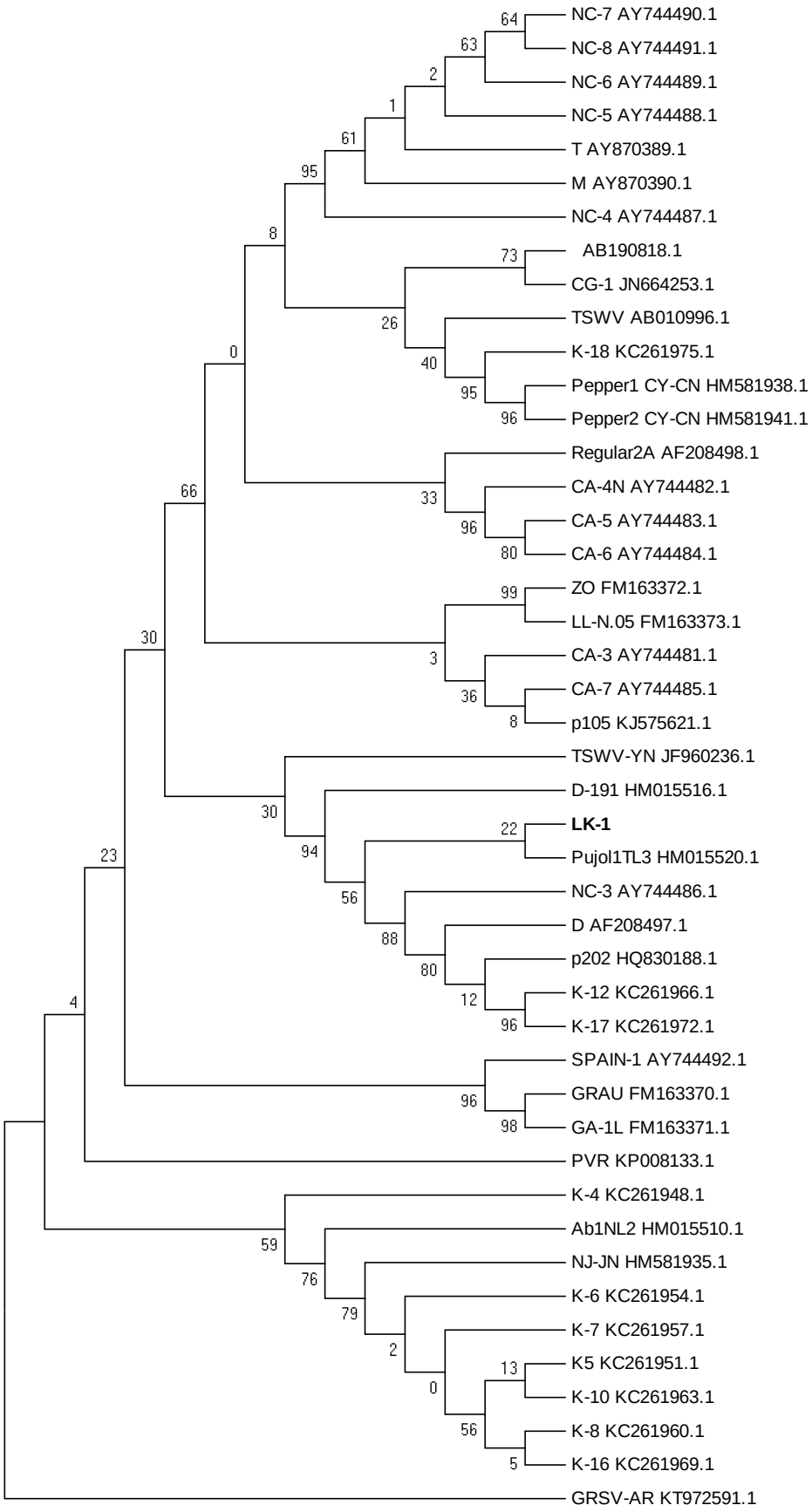


Figure 3.3: Phylogenetic relationship of the NSm sequences of the LK-1 TSWV isolate

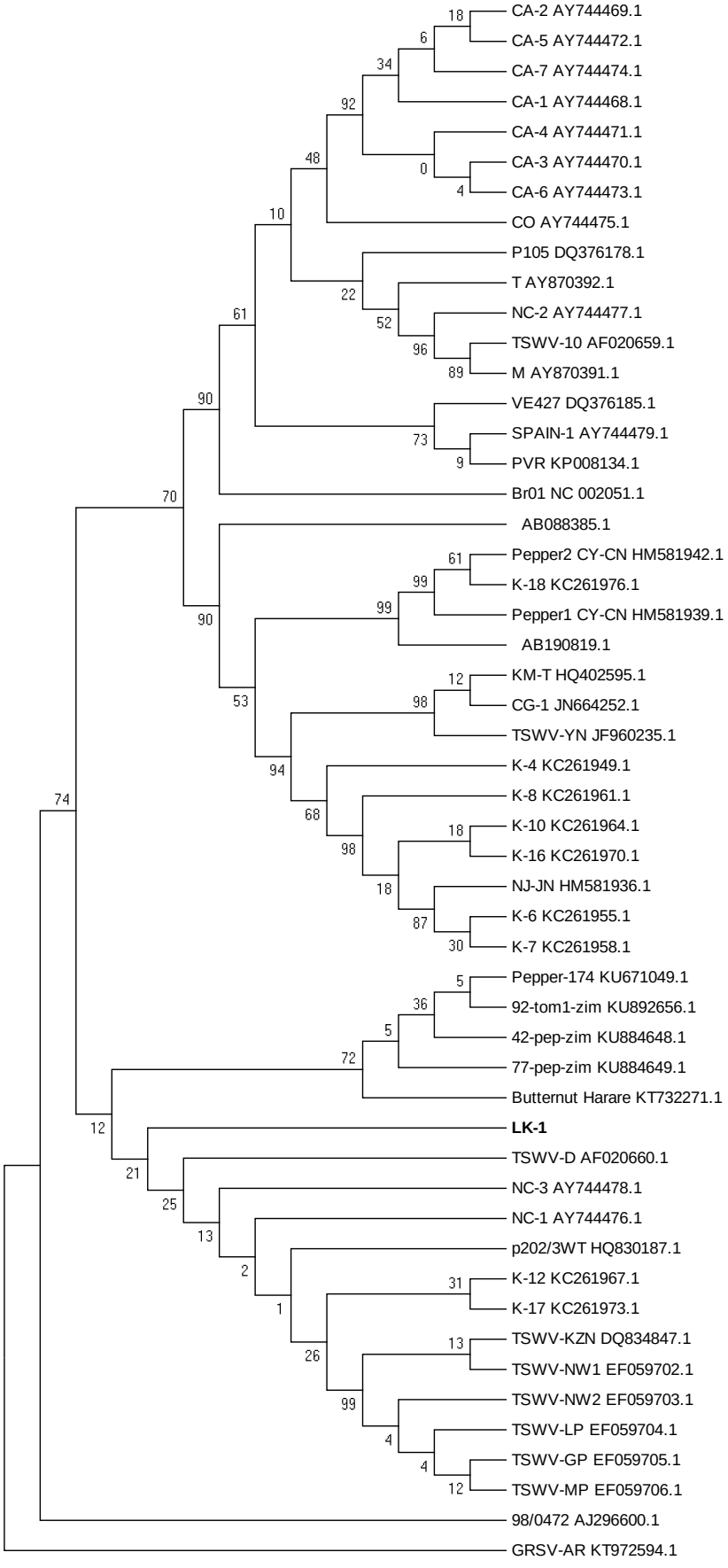


Figure 3.4: Phylogenetic relationship of the N sequences of the LK-1 TSWV isolate

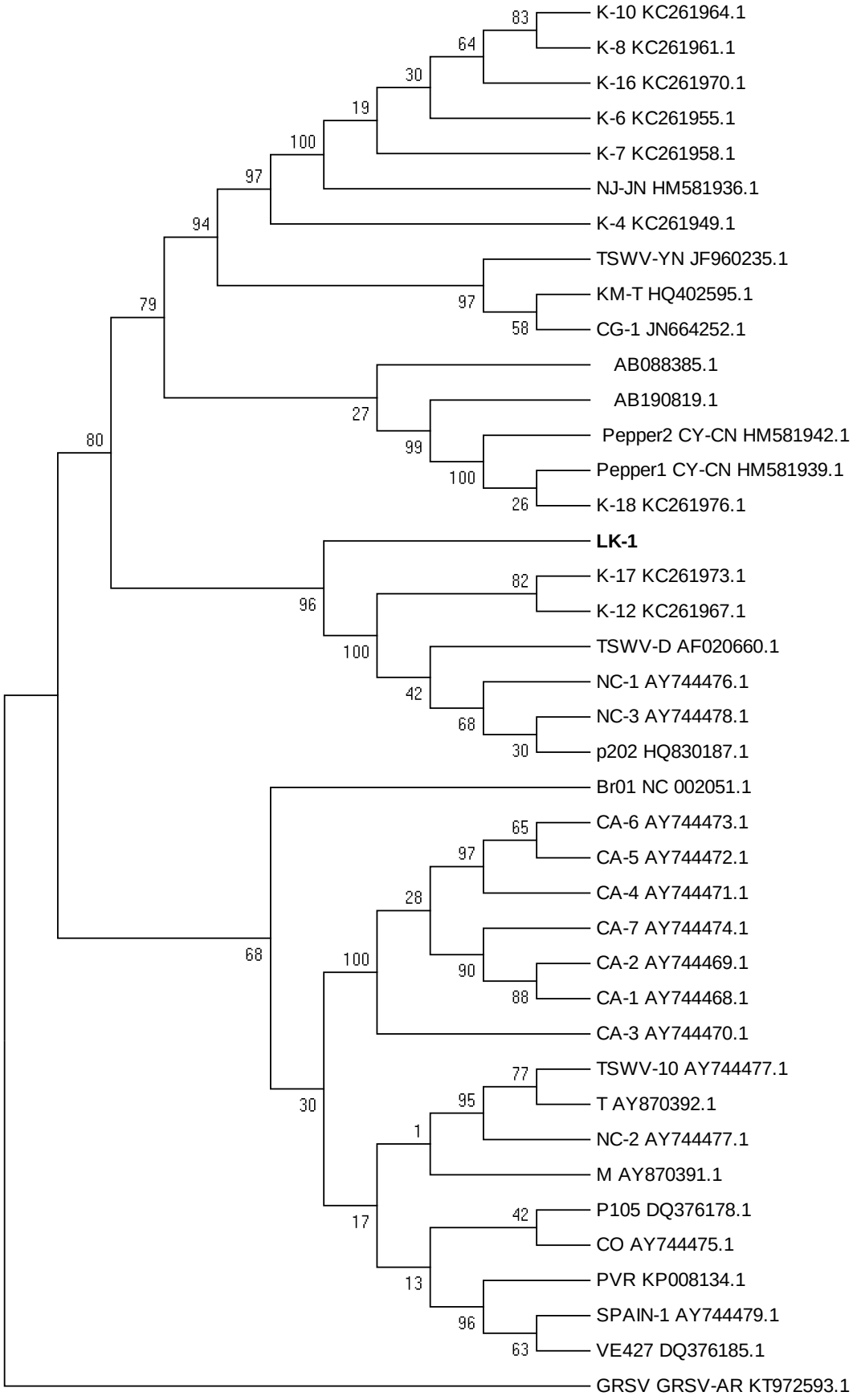


Figure 3.5: Phylogenetic relationship of the NSs sequences of the LK-1 TSWV isolate

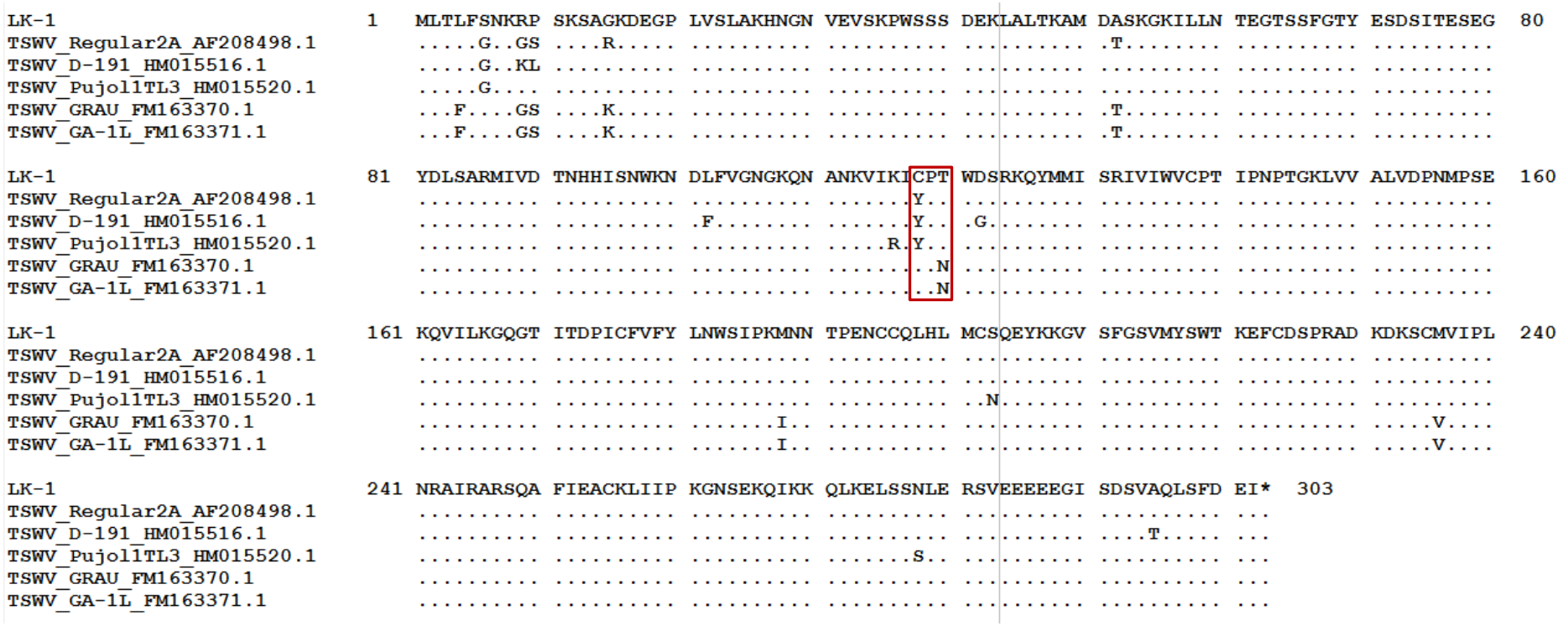
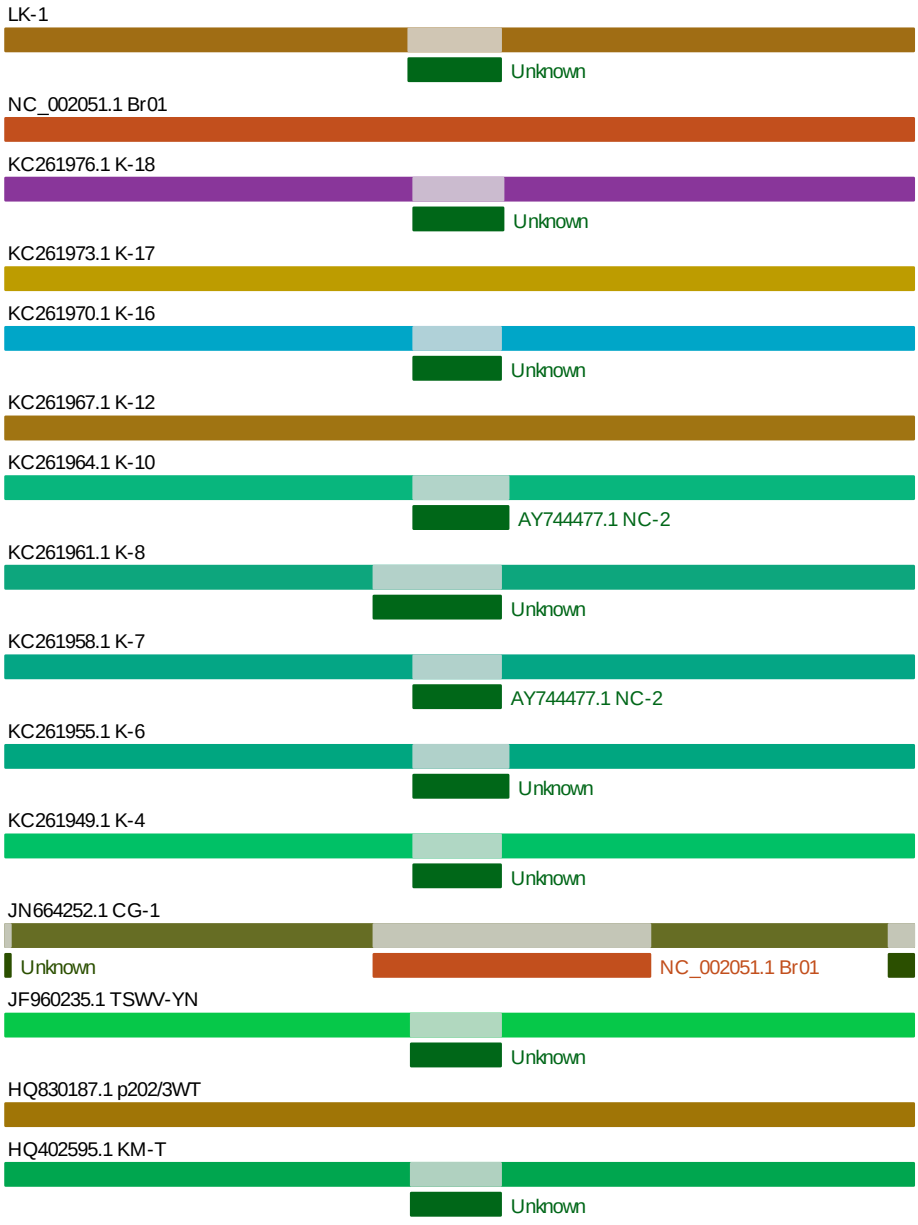


Figure 3.6: A mutation at position 118 or 120 on the TSWV NSm segment; a pathogenicity determinant against the *Sw-5* gene which confers resistance to TSWV in tomato crops.

3.4.5 Recombination analysis

No recombination events were observed on the L and M segments when tested with parental and recombinant TSWV isolates (data not shown). A similar result was observed on the S segment when tested with parental isolates (data not shown). However, when the S segment was assessed for recombination with other known recombinant TSWV isolates, one recombination event was observed (Fig. 3.7). The recombination event was confirmed by 3 methods i.e. RDP (5.499 $x10^{-08}$), Max Chi (2.574 x 10^{-08}), Chimaera (1.323 x 10^{-07}) implemented in RDP4. The event occurred at 1383 nt to 1711 nt and consists of a major parent C-G1 with 95.8% similarity to the isolate and an unknown minor parent.



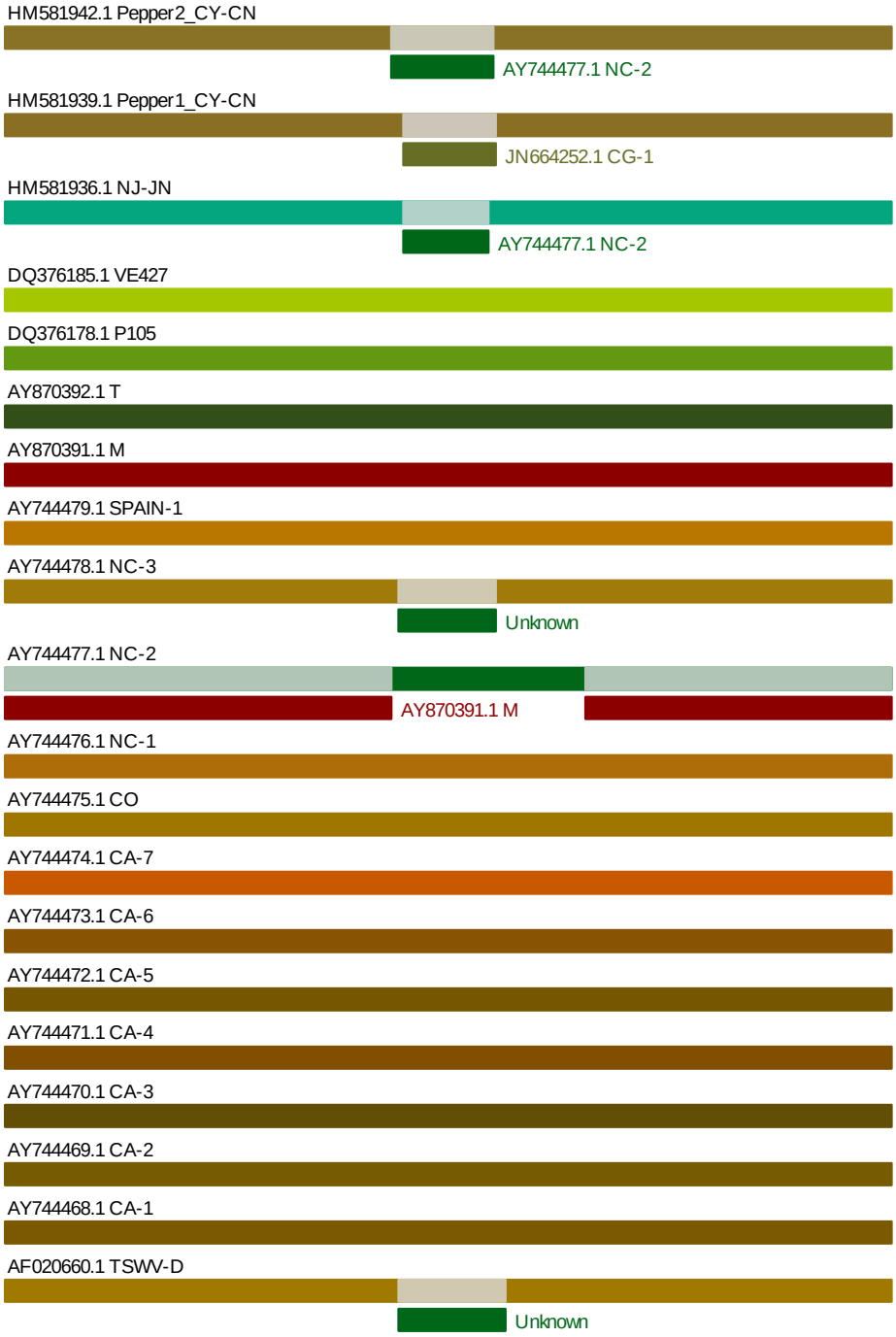


Figure 3.7: Recombination events occurring at one breakpoint of the sequence alignment for the South African TSWV isolate (LK-1) identified using RDP4 analysis. Recombinant TSWV isolates were used in conjunction with LK-1 in this analysis.

3.5 DISCUSSION

The genetic diversity of TSWV has been a predominant focus of virological studies in recent years. Despite TSWV's global economic importance, relatively few complete genomes for TSWV have been determined. The majority of the TSWV isolates have

only been partially characterised. This is the first report of a complete genome sequence of a TSWV isolate from South Africa.

TSWV symptoms and thrips were not widespread in South African farms for a considerable length of time. The last report of TSWV in South Africa was by Sivparsad and Gubba (2008). Crop losses due to viral diseases in South Africa were predominantly attributed to whitefly and aphid-borne viral diseases. However during the national survey conducted in this study high thrip populations were observed in the northern, eastern and central parts of South Africa. The TSWV isolates detected in this study were identified in the eastern part of the country (Chapter 2; section 2.4.2). It is very likely that TSWV may be present in the other regions where thrip populations are abundant. Therefore further analysis should be done. These results indicate that TSWV may be a re-emerging virus in South Africa.

It is likely that TSWV is difficult to mechanically transmit, as a result infection could not occur on inoculated plants. Therefore vector-mediated transmission may be more effective in transmitting the virus. This implies that the control of the thrip vector will be an essential component in an integrated pest management strategy to curb possible future TSWV outbreaks.

NGS technology has revolutionised plant virology. It efficiently facilitates the acquisition of complete viral genomes with relative ease (Kesanakurti *et al.*, 2016). NGS was the principal tool used in this study to obtain the full genome of TSWV. Three of the contigs generated from *de novo* assembly made up the complete genome of TSWV with each individual contig comprising of the L, M and S segment. BLAST analysis of the contigs matching to TSWV revealed an 88% to 97% sequence identity with other complete sequences of the segments of TSWV.

The genome organisation of TSWV LK-1 is characteristic of other TSWV isolates (Debreczeni *et al.*, 2015; Margaria and Rosa, 2015). The complete genome of TSWV LK-1 consists of 16 668 nt and the sizes of the L, M and S were 8912 nt, 4820 nt and 2936 nt respectively (Table 3.3). The RdRp, Gc-Gn, NSm, N and NSs ORFs were 8640 nt, 3408 nt, 909 nt, 777 nt and 1404 nt respectively (Table 3.3). The 5’ untranslated regions of the L, M and S segments were 33 nt, 100 nt and 189 nt respectively while the 3’ non-coding regions were 239 nt, 83 nt and 150 nt respectively.

The phylogenetic analysis of all 3 segments of TSWV showed no correlation between geographic location and genetic relationships. The geographically diverse clades observed suggests the possibility of numerous long-distance migration events (Zhang *et al*., 2016). The phylogenetic relationships among the South African TSWV isolate and other TSWV isolates were diverse for each ORF suggesting possible reassortment. Together with long-distance migration and recombination, the interchange of whole genomic segments plays a pivotal role in the evolution of TSVW (Margaria *et al.*, 2015; Margaria and Rosa, 2015). Phylogenetic analysis of LK-1 using complete sequences of the RdRp ORF showed genetic diversity as RdRp did not form a cluster with any of the parental TSWV isolates (Fig 3.1). While the Gc-Gn, N and NSs ORFs clustered with a geographically diverse array of isolates (Fig 3.2; 3.4; 3.5). These results indicate that different evolutionary lineages are present.

Although the N ORF of LK-1 formed a clade with other South African isolates, the bootrap values indicate that they are not closely related (Fig. 3.4). This suggests that LK-1 is a different TSWV isolate than those previously reported in South Africa. A divergence of Zimbabwean isolates was observed from LK-1 (Fig. 3.4). However, only partial sequences were available from the South Africa and Zimbabwean isolates. Full genomes of these isolates will provide a comprehensive understanding of southern African TSWV evolution.

Interestingly the NSm ORF showed to be closely related to two *Sw-5* resistance breaking isolates, the Australian D-191 isolate and the Spanish Pujol1TL3 isolate (Fig. 3.3; López *et al.*, 2011; Debreczeni *et al.*, 2015). Studies show that the substitutions of a Cys amino acid residue with a Tyr residue at codon 118 or Thr with Asn at codon 120 in NSm protein of TSWV is implicated in breaking down the *Sw-5* resistance gene (Aramburu *et al.*, 2010; Debreczeni *et al.*, 2015). Reports of resistance breaking isolates in crop fields are on the rise (Margaria *et al.*, 2015). This may be due to enhanced selection pressure due to the extensive use of tomato plants with the *Sw-5* gene (López *et al*., 2011). Although LK-1 clustered closely with the *Sw-5* resistance breaking isolates D-191 and Pujol1TL3, it did not have any point mutations that are typically associated with *Sw-5* resistance breaking (Fig. 3.3; Fig. 3.6). Therefore LK-1 is a *Sw-5* non-infecting isolate (López *et al*., 2011). This may explain why no symptoms were observed on inoculated tomato crops. Furthermore, it is unlikely that the LK-1 isolate will break down the *Tsw* resistance in pepper

because it does not cluster with the *Tsw* resistance breaking isolate LL-N.05 (Fig 3.1; 3.2; 3.3; 3.4; 3.5). This is consistent with no symptom development observed on any of the inoculated plants. The amino acid substitution associated with overcoming the *Tsw* resistance is unknown at this stage (Debreczeni *et al.*, 2015).

RDP4 analysis did not show any recombination for the L and M segments (data not shown). However, one recombination event was observed on the S segments when assessed for recombination using other recombinant TSWV isolates (Fig. 3.7). LK-1 had recombined with the Chinese isolate C-G1. These results suggest that the C-G1 isolate may have been introduced to South Africa through imports from China. It was noted that the majority of the recombination events of all the isolates tested occurred at the same point (Fig. 3.7). The majority recombination events on the S segment took place between 1383-1711 nt which is part of the NSs ORF and the 5' untranslated region of the S segment, however, some did occur at the 3' and 5' proximal region. This implies that the majority of recombinations events are limited to this region. This is consistent with reports by Zhang *et al.*, 2016.

TSWV is a constantly evolving pathogen. Co-infection or viral synergism of TSWV and other pathogens result in heightened symptom development and has the potential to cause devastating crop losses. Therefore, the use of molecular analysis will improve the understanding of how TSWV is evolving and the rate at which it evolves. This information is vital to the effective management of this pathogen in the face of climate change and pesticide abuse.

3.6 CONCLUSION

The complete genome sequences of a South African TSWV isolate will contribute to the understanding of the emergence and evolution of TSWV and its adaptation to new hosts. The phylogenetic analyses on TSWV isolates worldwide suggest possible reassortment of the genomic segments. The findings of this study are essential in the development of effective management strategies of TSWV. Sources of natural TSWV resistance from wild or native plants should be evaluated. In addition, early warning systems and monitoring programmes of TSWV will help reduce potential outbreaks.

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CHAPTER FOUR

DISSERTATION OVERVIEW

A weed can be defined as a plant growing in an area where it is not wanted. Weeds are often considered undesirable components of agricultural cropping systems. They are widely known for their proclivity for acting as pests themselves by outcompeting crop plants for essential nutrients, space, water and sunlight. However, studies have shown that weeds also have the propensity to harbour viral pathogens and the insect vectors that transmit them. The research undertaken in this dissertation was initiated with the intention to increase the knowledge of the weed species harbouring specific viruses in South African crop fields and in addition contributing to the global awareness of weed reservoirs of viral pathogens. Next-generation sequencing (NGS) is a new technique that is widely being used in molecular biology and virology to obtained complete genomes of viral pathogens due to its high throughput potential and efficiency in sequencing full genomes. Therefore in this study NGS was used to characterise the genome of a South African *Tomato spotted wilt virus* (TSWV) isolated from *Amaranthus thunbergii*.

4.1 MAJOR FINDINGS

In chapter two four distinct viruses were detected on four different weed species; TSWV on *A. thunbergii*, *Potato virus Y* (PVY) on *Physalis peruviana*, *Tomato chlorosis virus* (ToCV) on *Solanum nigrum* and a mixed infection of *Tomato torrado virus* (ToTV) and ToCV was detected on *Datura stramonium*. These viruses were also detected on tomato crops adjacent to the weeds samples. *A. thunbergii* and *P. peruviana* were reported as new hosts of TSWV and PVY, respectively, in South Africa (Kisten *et al.*, 2016a; Kisten *et al.*, 2016b). These results suggest that weeds have the ability to harbour vectors and vector-borne viral diseases and as a result facilitate the virus infection between weed and crop via insect vectors.

In chapter three the first complete genome sequence of a South African TSWV isolate (LK-1) was reported. Phylogenetic analysis of the TSWV genomic segments revealed that LK-1 is a unique South African TSWV isolate. Furthermore,

phylogenetic analysis also showed that the NSm ORF on the M segments is closely related to two *Sw-5* resistance breaking isolates, isolates D-191 and Pujol1TL3. However, inspection of the amino acids substitution involved in the breakdown of the *Sw-5* resistance revealed that the point mutation was not present in the LK-1 isolate. Recombination analysis detected one recombination event on the S segment that showed apparent regional preference to the NSs and 5' untranslated region of the S segment.

4.2 WAY FORWARD

An extensive bioassay of other Solanaceae crops should be conducted to determine the pathogenicity and host range of the viral isolates detected on weed species in this study. Different inoculation techniques should be tested such as vector-mediated transmission or infectious clones. This will further substantiate reports that weeds act as reservoirs of virulent viral pathogens. Due to the wide distribution of the weeds tested in this study in South Africa, other weeds species should also be tested for viral infection. Furthermore, additional isolates of the four viruses detected in this study should be sequenced. This will provide a comprehensive population study of the viruses affecting weeds in South Africa. Early warning systems and monitoring programmes should be developed to prevent plant viral outbreaks in South Africa.

Full genome sequences of other South African TSWV isolates should be obtained. This will give a clearer indication of possible recombination or reassortment between South Africa TSWV isolates. Lastly, the other viruses briefly mentioned in this study that were detected by NGS in the *A. thunbergii* sample should be subjected to further genetic analysis.

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APPENDIX ONE

COMPLETE GENOME OF *TOMATO SPOTTED WILT VIRUS*

1. NUCLEOTIDE COMPOSITION OF LK-1 LARGE SEGMENT

1 AGAGCAATCAGGTAACAACGATTTTAAGCAAACATGAACATCCAGAAAATACAAAAATTA

61 AAATGGAACCACTTTACTGTTGTCTATTGAGGATTGTGTAGGTTCTAACCACGATCTAGC

121 TTTGGATTTACATAAGAGAAATAGTGATGAGATCCCAGAAGATGTGATTATAAATAATAA

181 TGCAAAAAATTATGAGACAATGAGAGAGTTAATTGTCAAAATCACTGCTGATGGTGAAGG

241 ACTAAACAAAGGGATGGCAACTGTGGATGTCAAAAAGCTAAGTGAGATGGTCTCTCTGTT

301 TGAGCAAAAATACCTAGAAACAGAGTTAGCAAGGCATGACATTTTTGGAGAGCTGATCTC

361 CAGGCACCTGAGAATAAAGCCCAAACAAAGAAATGAAGTGGAGATAGAGCATGCACTAAG

421 AGAATATCTGGATGAACTCAACAAAAAGTCCTGCATTAACAAGCTCTCTGATGATGAGTT

481 TGAGAGAATAAATAAAGAATATGTAGCAACTAATGCCACCCCTGATAACTATGTGATATA

541 TAAAGAATCAAAAAACAGTGAGCTTTGTTTAATCATTTATGATTGGAAAATATCTGTCGA

601 TGCCAGGACTGAAACCAAACAATGGAGAAATACCTACAAGAATATTTGGAAATCTTTCAA

661 AGATATAAAAGTGAATGGAAAGCCATTCCTGGAAGAGCATCCTGTTTTCGTTTCTATAGT

721 TATATTGAAACCTATTGCTGGGATGCCAATCACTGTTACTAGTAGCAGGGTTTTGGAGAA

781 ATTCGAAGATTCTCCATCAGCATTGCACGGAGAAAGAATAAAGCATGCTAAAAATGCCAA

841 ATTGCTAAATATTTCTTATGTTGGGCAAATAGTTGGAACCACACCCACAGTGGTGAGAAA

901 CTATTATGCAAACACTCAAAGAATCAAATCTGAAGTCAGAGGAATCTTAGGTGATGATTT

961 TGGATCTAAAGATGTGTTTTTCAGTCACTGGACCAGCAAATACAAAGAAAGAAATCCTAC

1021 TGAGATAGCCTATTCCGAAGATATTGAAAGAATAATTGATTCACTTGTTACAGATGAAAT

1081 CCCTAGAGAGGAAATAATACATTTTTTGTTTGGAAATTTCTGTTTCCACATTGAAACAAT

1141 GAATGACCAGCATATAGCTGACAAATTTAAAGGGTACCAAAACTCTTGTATCAATTTAAA

1101 AATAGAGCCAAAAGCTGATTTAGCTGATTTGAAAGACCACTTAATCCAAAAGCAGCAAAT

1161 ATGGGAATCTCTGTATGGAAAACACCTTGAGAAGATCATGCTTAGAATTAGAGAAAAAAA

1201 GAGAAAAGAAAAAGAGATACCTGACATAACCACAGCTTTTAACCAGAATGCTGCTGAATA

1261 TGAAGAAAGGTATCCTAACTGTTTCAATGATCTCTCTGAACTAAAACTAACTTTCCATGA

1321 CTTGGTCCCCAGTTTGAAGATAGAATTGAGCTCAGAGGTAGATTACAACAACGCAATTAT

1381 TAACAAGTTTCGGGAGAGCTTCAAAAGTTCTTCAAGGGTTATTTATAATAGCCCATATAG

1441 TAGCATAAATAACCAAACAAATAAAGCAAGAGATATAACAAACTTAGTTAGACTGTGTTT

1501 AGCAGAGCTAAGTTGTGATACAACGAAAATGGAAAAGCAGGAACTTGAAGATGAAATAGA

1561 TATAAACACCGGGAGTATCAAAGTTGAGAGAACAAAAAAGTCTAAAGAATGGAATAAGCA

1621 AGGTTCGTGTTTAACCAGAAACAAAAATGAATTTTGCATGAAAGATACAGGCAGGGAGAA

1681 CAAAACTACCTATTTTAAAGGCTTAGCAGTAATGAATATAGGAATGAGTTCTAAGAAAAG

1741 AATTCTAAAAAAAGAAGAAATAAAAGAAAGGATCTCTAAAGGCTTGGAATATGATACCTC

1801 TGAAAGGCAGGCTGACCCAAATGATGATTACTCAAGTATAGACATGTCTTCTCTGACTCA

1861 TATGAAAAAACTGATAAGGCATGACAATGATGATAGCTTAAGTGGTAAAAGATTTAAGGG

1921 CTCTTTTTTTCTACTTCATAATTTTAATATAATAGAGGATGGTAAGATCACATCTGTTTT

1981 CAATAATTATGCTAAAAATCCTGAATGCTTGTACATTCAAGATTCAGTACTGAAGACTGA

2041 ATTAGAGACTTGCAAAAAGATAAACAAATTATGCAATGATCTAGCCATTTACCATTACTC

2101 TGAAGACATGATGCAATTCTCCAAAGGTTTAATGGTGGCTGACAGGTACATGACTAAAGA

2161 AAGTTTCAAGATATTAACCACAGCAAATACTAGCATGATGCTATTAGCATTCAAAGGGGA

2221 TGGAATGAACACCGGAGGATCGGGAGTTCCTTACATAGCATTGCATATAGTGGATGAAGA

2281 CATGTCAGATCATTTTAACATATGTTATACTAAAGAAATTTATAGCTATTTCCGAAGTGG

2341 TAGTAATTACATTTATATAATGAGGCCGCAGAGACTAAACCAGGTGAGGCTGCTGAGGCT

2401 TTTCAAAACGCCTAGTAAAGTTCCTGTATGTTTTCCACAATTTTCAAAGAAAGCTAATGA

2461 AATCGGAAAATCGCTGAAAAATAAAGATATAGAAAAAGTAAATCTCTTTTCTATGACAAT

2521 GACTGTAAAACAGATATTAATAAATATTGTGTTTTCATCTGTCATGATAGGAACTGTGAC

2581 AAAGCTCAGTAGAATGGGAATTTTTGATTTCATGCGGTATGCAGGTTTTTTGCGACTATC

2641 CGATTATTCTAACATAAAAGAATACATTAGAGACAAATCTGATCCTGATATAACTAACTG

2701 TGGCAGATATCTATTTCGTAATGGAATCAAAAAACTATTGTTCAGAATGGAAGATCTCAA

2761 TTTAAGCACAAATGCCAAGCCTGTTGTTGTGGACCACGAAAATGATATTATAGGAGGGAT

2821 AACAAACTTGAATATAAAATGTCCTATAACAGGATCAACTCTACTGACACTTGAGGACCT

2881 GTACAATAATGTTTATTTGGCTATTTATATGATGCCTAAATCACTGCACAATCATGTTCA

2941 CAATCTAACAAGCTTATTAAATCTCCCTGCTGAGTGGGAGCTAAAGTTCAGAAAAGAATT

3001 AGGTTTCAACATATTTGAAGACATATACCCTAAGAAAGCAATGTTTGATGACAAAGACCT

3061 ATTCTCCATAAATGGAGCTTTGAACGTGAAAGCATTATCTGATTACTATCTAGGAAATAT

3121 AGAAAATGTTGGTTTAATGAGATCAGAAATAGAAAATAAAGAAGATTTCCTAAGCCCTTG

3181 TTATAAAATATCTACTTTAAAATCTTCAAAAAAATGCTCACAATCAAACATTATAAGTAC

3241 TGATGAGATAATAGAGTGTCTTCAGGATGCAAAGATTCAAGATATAGAAAATTGGAAAGG

3301 AAATAACCTAGCTATTATAAAAGGGCTTATAAGAACCTACAATGAGGAGAAAAATAGATT

3361 GGTGGAATTTTTTGAAGATAATTGTGTCAATTCATTATATCTTATAGAAAAGCTTAAAGA

3421 GATAATTAGTAGTGGATCAATAACTGTAGGGAAATCTGTAACATCTAAATTCATAAGAAA

3481 CAATCATCCTTTAACAGTAGAAACATATCTCAAAACAAAACTATATTATAGAAATAATGT

3541 AACAGTTTTAAAGTCTAAAAAAGTGTCAGAGGAGCTCTATGACCTTGTAAAACAGTTCCA

3601 TGACATGATGGAAATAGACCTAGATTCTGTTATGAACCTCGGGAAAGGTACAGAAGGAAA

3661 AAAACTCACATTCTTGCAGATGCTTGAATTTGTCATGTCCAAGGCTAAAAATGTCACCGG

3721 GTCTGTAGATTTTCTAGTTTCTGTTTTTGAAAAAATGCAGAGAACCAAAACAGACAGAGA

3781 AATATACTTGATGAGCATGAAAGTGAAAATGATGCTTTATTTTATAGAGCATACATTCAA

3841 ACATGTAGCGCAGAGTGATCCATCGGAAGCCATATCTATAAGTGGAGACAATAAAATAAG

3901 AGCACTTTCTACGTTATCTTTGGACACAATCACGTCTTACAATGATATTTTAAACAAAAA

3961 TTCAAAGAAGTCAAGATTGGCTTTCCTATCTGCAGATCAGTCGAAATGGTCGGCATCAGG

4021 CCTTACCACCTATAAATATGTTTTAGCTATCATATTAAATCCAATTTTAACTACTGGTGA

4081 AGCTAGCTTAATGATAGAATGCATCTTAATGTATGTTAAATTGAAGAAGGTTTGTATACC

4141 AACAGATATTTTTTTGAATCTAAGAAAAGCTCAACAAACTTTTGGGGAAAATGAAACTGC

4201 CATAGGACTTTTGACCAAAGGCTTGACGACAAACACATACCCTGTTAGCATGAATTGGTT

4261 GCAAGGCAATTTAAATTATCTGTCTTCTGTTTATCACTCTTGTGCAATGAAAGCTTATCA

4321 CAACACTTTGGAATGTTACAAAAACTGTGATTTCCAAACTAGATGGATTGTGCACTCTGA

4381 TGATAATGCAACATCATTAATAGCCAGTGGAGAGGTTGATAAAATGCTGACAGACTTTTC

4441 AAGCTCATCTCTGCCAGAAATGTTGTTTAGAAGCATTGAAGCTCATTTCAAAAGTTTTTG

4501 CATAACTTTGAACCCAAAAAAGAGTTATGCTTCTTCATCAGAAGTAGAGTTTATATCTGA

4561 AAGAATTAGTAAATGGAGCGATTATTCCTCTCTATTGCAGGCATTTAGCAAACTGTTGCA

4621 CAGAATCTTCGCATATAAGTTATTTGATGATCTAATGTCACTCAGTATACATGTTACAAT

4681 GCTTCTGAGAAAAGGCTGTCCTAATGAAGTTATACCTTTTGCTTATGGGGCTGTGCAGGT

4741 ACAAGCGTTAAGCATCTATTCAATGCTTCCTGGTGAAGTGAATGATAGTATTAGAATTTT

4801 TAACAAGCTTGGAGTAAGTTTAAAGTCAAACGAGATTCCCACAAACATGGGGGGCTGGTT

4861 GACCTCTCCTATAGAGCCGTTGTCTATATTAGGTCCATCATCAAATGATCAAATCATCTA

4921 TTACAATGTGATAAGAGATTTTTTGAACAAAAAAAGTTTAGAAGAAGTAAAAGATAGTGT

4981 CTCTTCTTCCAGTTATCTACAGATGAGATTCAGAGAGCTAAAAGAAAAGTATGAAAGAGG

5041 AACTCTGGAAGAAAAAGATAAAAAGATGATATTTCTTATCAATCTGTTTGAGAAAGCATC

5101 AGTGTCTGAAGATTCAGACGTTCTAACAATTGGGATGAAATTTCAAACTATGTTAACTCA

5161 GATTATAAAATTACCTAATTTTATAAATGAGAATGCTTTAAACAAGATGTCAAGTTATAA

5221 AGATTTTTCAAAACTTTATCCTAATTTAAAAAAGAATGAAGATTTATATAAAAGCACTAA

5281 GAACTTAAAGATAGACGAGGATGCTGTTTTAGAGGAAGATGAGTTATATAAGAAGATTGC

5341 ATCTAGCTTAGAAATGGAATCTGTCCATGACATAATGATAAAAAATCCTGAAACAATTCT

5401 GATAGCACCATTGAATGATAGAGATTTTTTACTTAGTCAGCTGTTCATGTACACAAGCCC

5461 TTCTAAAAGAAACCAGTTATCGAACCAATCTACAGAGAAACTTGCTTTAGATAGAGTGTT

5521 AAGGTCAAAAGCTAGAACATTTGTAAACATTTCTTCCACTGTGAAGATGACTTATGAAGA

5581 AAACATGGAAAAGAAAATCTTAGAAATGCTAAAATTTGATTTAGATTCATATTGTTCATT

5641 TAAAACATGTGTAAATCTAGTTATCAAGGATGTTAATTTCAGCATGCTGATTCCAATATT

5701 AGATTCTGCATACCCTTGTGAATCTAGGAAAAGAGATAACTACAATTTCAGGTGGTTTCA

5761 GACTGAGAGATGGATACCTGTTGTTGAAGGCTCTCCGGGACTAGTAGTAATGCATGCTGT

5821 CTATGGATCAAATTATATAGAGAACTTAGGTTTAAAAAACATCCCTCTAACAGACGATAG

5881 TATTAATGTTTTAACAAGCACGTTTGGAACAGGTTTAATCATGGAAGATGTAAAATCCCT

5941 AGTTAATGGCAAAGACAGCTTCGAAACAGAGGCTTTTAGCAATTCTAATGAATGTCAAAG

6001 ATTGGTGAAAGCATGCAATTATATGATAGCAGCACAAAACAGGCTTTTAGCAATTAACAC

6061 ATGCTTTACTAGGAAAAGCTTTCCCTTCTATTCTAAGTTCAATCTAGGGAGAGGGTTTAT

6121 CTCAAACACATTAGCTCTCCTATCCACCATCTACAGTAAAGAAGAATCCTATCATTTTGT

6181 TTCTACAGCTAGTTATAAATTAGACAAAACTATCAGAACTGTGGTAAGTGCTCAGCAAGA

6241 TATGAACTTAGAGAAAATACTGGACACTGCTGTATACATATCAGATAAATTGCAGTCACT

6301 TTTCCCAACAATTACAAGAGAGGATATAGTTTTAATATTGCAAAATGTTTGCCTTGACAG

6361 TAAACCTATATGGCAGAGTCTAGAAGACAAAATGAAAAAGATTAACAATTCAACAGCAAG

6421 TGGCTTCACAGTGTCAAATGTGATTCTATCACATAACAGTGAATTGAACACAATCCAGAA

6481 ACAAATTGTCTGGATGTGGAACATGGGTTTGTGTTCTCACAGAACATTAGATTTTGTTAT

6541 CAGGTATATTAGAAGAAGGGATGTAAGATATGTAAAAACTGAAGAACAAGATGAATCAGG

6601 AAATTATGTCTCTGGAACTATGTACAAAATAGGGATCATGACAAGAAGCTGCTATGTGGA

6661 ATTGATAGCATCTGATCAAGATGTAGCAGTTTCTTTGAGAACACCATTTGAGATATTGAA

6721 TGAAAGAGAGTATCTTTTTGACACATACAGAGAAAGTATAGAGAAATTATTGGCAGAAAT

6781 TATGTTTGATAAAGTGAACATAATAAATCAAACAACCACAGATTGTTTTCTTAGAACCAG

6841 GAGATCTTGCATCAGAATGACCACAGACAACAAAATGATTGTAAAGGTTAATGCTACATC

6901 AAGACAAATAAGACTAGAGAATGTAAAATTAGTTGTAAAGATAAAATATGAAAATGTGAA

6961 TTCCGATGTATGGGATATTATAGAAAGCCAAAAATCTCTAGTCTTAAGGCTCCCTGAAGT

7021 AGGGGAATTTTTCTCTGATATGTATAAAACTGCAGACTCTGAAACTGAAACAATCAAAAC

7081 CATAAAAAACAGGCTTATGACTTCTTTAACTTTCATAGAAGCCTTTGGAAACTTATCACA

7141 GCAGATCAAAGAGATTGTAGATGATGATATCAGAGAAACGATGGATGAATTCTTAATGAA

7201 CATCCGGGATACCTGCTTAGAAGGTTTGGAAAACTGCAAAAGTGTGGAAGAATATGATAG

7261 CTATCTTGATGAAAATGGATTTAATGACACAGTAGAACTATTCGAAAACTTGCTAAGAAC

7321 ACATGACAACTTTGAAAATGAGTATAGTCCTCTTTTTTCAGAGATTGTTGACAAAGCAAA

7381 ACAGTATACTAGAGATTTAGAAGGTTTCAAAGAAATACTGCTCATGCTTAAATATTCTCT

7441 AATAAATGATGCATCAGGATTTAAAAGCTATAGAGCCACTGGAATGCATGCTGTTGAGCT

7501 AATGGCAAAAAAGCACATAGAGATAGGGGAATTCAACTTGTTAGGAATGATCCAATTGAT

7561 TAAAGCTTGTGAAACATGCCACAACAATGACTCTATATTAAACTTAGCAAGTTTAAGGAA

7621 TGTTCTTAGCAGGACATATGCCACATTTGGGAGGAGAATAAGATTGGATCATGATCTGGA

7681 CTTGCAAAACAACTTAATGGAAAAAAGTTATGATTTCAAGACGCTGGTTTTACCAGAAAT

7741 AAAATTATCAGAACTATCTAGGGAAATACTGAAAGAAAATGGGTTTGTTATATCTGGAGA

7801 GAATCTAAAAATGGATAGGTCTGATGAAGAATTTGTGGGTCTTGCCAGTTTTAATGTGTT

7861 GAGGCTAGATGAGGAAGAAATGTATGAAGGTTTGATCAAAGAAATGAAAATTAAAAGGAA

7921 AAAGAAAGGGTTTTTATTTCCAGCAAACACACTTCTACTAAGTGAGTTGATAAAGTTCTT

7981 GATTGGAGGAATAAAGGGAACCAGCTTTGATATAGAGACATTGTTACGGAACAGTTTTAG

8041 ACCAGACATATTTTCAACTGACAGATTGGGAAGATTAAGTTCCAGTGTACCTGCACTCAA

8101 AGTTTATGCAACTGTTTATATGGAATATAAGAATGTCAATTGTCCTTTAAATGAGATAGC

8161 TGACAGCTTAGAAGGTTATCTAAAACTGACAAAAAGCAGGTCCAAGGAACATTTCTTGTC

8221 TGGAAGAGTTAAAAAAGCTTTGATACAATTAAGAGATGAACAATCGCGAACTAAAAAACT

8281 AGAGGTCTATAAGGATATCGCAAATTTCCTTGCTAGGCACCCACTATGTTTATCAGAAAA

8341 AACATTGTATGGAAGATATACCTACTCTGATATCAATGATTATATCATGCAAACAAGAGA

8401 GATTATTTTGAGTAAAATAAGTGAGTTGGACGAGGTTGTTGAAACAGATGAAGACAATTT

8461 CTTGCTTAGTTATCTAAGAGGGGAAGAAGATGCCTTTGATGAAGATGAGCTTGATGAAGA

8521 AGAAGACACAGATTAAATTGAAAGTAATGACTAACAATCCATGAATAACAGATTAGATAT

8581 AACTTAGAATATAAATTTATTGCTATTTTAGAATTAGATTAGATCTACTTAGCCTAAAAC

8641 AATTTGGTGAACCAAATCTATAGTGTATATAAATGTAGAGTCCCGGTATAGTTTCACTGG

8701 AGGGAATTCTTATGTAATTTGTAAAGTCTGGCTGTGGAGAGGTTATATGTTTTAGTTGTA

8761 CCTGATTGCTCTGAGGGGAAGAAGATGCCTTTGATGAAGATGAGCTTGATGACTTGCTTA

8821 GTTATCTAAGAGGGGAAGAAGATGCCTTTGATGAAGATGAGCTTGATGAAGAAGAAGACA

8881 CAGATTAAATTGAAAATTAAGAGATGAACAA

2. NUCLEOTIDE COMPOSITION OF LK-1 MEDIUM SEGMENT

1 AGAGCAATCAGTGCATCAGAAATATACCTATTATACATTTTGCTAAGAATCAATCAACTA

61 CATTACACAAGCTCCTCTACCTTAGGCTGTTGAACTCGAAATGTTGACTCTTTTCGGTAA

121 CAAGAGGCCTTCTAAGTCTGTAATGGCAGTGTTGAAGTCTCAAAACCATGGTCTTCTTCT

181 GATGAAAAGCTTGCTTTAACCCGGAAAGGATGAAGGTCCTTTAGTTTCACTTGCTAAACA

241 CAAAGCCATGGATGCATCCAAAGGAAAGATACTGTTAAACATTGAGGGAACATCTTCCTT

301 TGGAACCTATGAATCTGATTCCATCACAGAGTCAGAAGGTTATGATCTTTCTGCTAGAAT

361 GATAGTAGATACAAACCATCATATCTCAAACTGGAAAAATGATCTTTTTGTTGGCAACGG

421 AAAGCAAAATGCTAATAAGGTTATCAAGATCTGTCCGACTTGGGACAGCAGAAAACAATA

481 CATGATGATTTCCAGAATTGTGATATGGGTCTGCCCCACTATACCAAACCCTACAGGAAA

541 ACTTGTGGTTGCTTTAATTGATCCCAACATGCCATCTGGAAAGCAAGTCATCCTGAAGGG

601 TCAGGGGACAATAACTGATCCTATCTGCTTTGTTTTTTATCTGAACTGGTCTATTCCGAA

661 GATGAACAACACCCCAGAAAACTGTTGTCAGCTGCATTTGATGTGTAGCCAAGAATACAA

721 GAAAGGGGTTTCTTTTGGTAGTGTCATGTATTCTTGGACAAAAGAGTTTGGTGATTCACC

781 CAGAGCTGATAAAGACAAAAGTTGTATGGTTATACCTCTAAACAGGGCCATTAGAGCTAG

841 ATCTCAGGCATTCATTGAAGCCTGCAAGCTGATAATTCCTAAAGGAAACAGTGAGAAGCA

901 GATAAAAAAACAGCTTAAAGAACTGAGCTCAAATCTTGAGAGATCAGTTGAAGAGGAAGA

961 GGAAGGAATTTCTGACAGTGTTGCTCAGTTATCCTTTGATGAAATATAGTTCTTTAAATA

1021 TCACTTATTTAAGCTTAAATTTCTGTCTATTTTGCATTTTGAATCCAAAAACCCAAACAA

1081 AAAACGAAAACAAAAAAGAGAAAAAAACAAAAAATCAAACCAAAAAACAAAAACAAAATA

1141 AGGCTGAAAAGCAAACTTTGGTCCGAAGACTTTTTTTGTTGTTTTTTGTTTATTTTTATT

1201 TTTTTGTTTGTTTTTTGTTTATTTTATATTTCGTTTTTATTAGTTAATGATTGATTTTAA

1261 AGATTTTTCTATATATATAATCCTGCTAATATAGAAGATTGAATCAAATTTAATCTGTGA

1321 CAAGCATCTTCAGACAAGGTGAGAGAAATCCATAGGTGGCCTTCTTCTTGTCATTGTATC

1381 TTTCATTAACATAGGGGCTTTGATCTCAGGTTCATCATCATCCTCTATCTTGGATCTAGA

1441 TTTATAAGATTCATTCTTTACATATCCTTTACAAATGGATGTCAGAATAGAACAGAAATA

1501 AGTCACAAGAAAAATGAATGCAATAAGCAGTACCACTCTGATAGTATCAAAAAATGAGCC

1561 AAAGTAACTTGCAATGAAATTGAATGGACTCTTAATATAATCCCAGAAGCCCCATGCTGA

1621 AGAATCAGAATTATATTGTTGTTCTTCATGAGCATACTCATCATTTTGATCTATTATATT

1681 CTCTGGTTCTTCTACAATAACATTATTAACCAAAACTTCCACAGAGATATCCGGATTGCC

1741 TTCTGGATACAATGTCATTTTCTTCTTGTCCGGATTGGCTGAACAAAACATTGTTATATT

1801 GTATTTATTAGATCCTTTTTTAACAGCTAGCTGATAAGTAGATAAAGAGCAAGCATCTAT

1861 AGAAATTGCAGTAGAAAATGTCAAATCTGAGAAAAATTCTAAAAGGCAAGATAAACCTTG

1921 GCCGCATAGAAGACAGCCATTGCAATTTAAGCTTGTTGAAGTTATGGAAGGTTTCTTAGG

1981 AGCAACTTTAAAAAGATCAGATGGAAGATCAACTACCATTTTCAGTTTCCCTAGACTAAA

2041 AGATTTTGCCAGAAAAAAACTAGAAAAATCTTTGAAACTAACAGGAATATCTGATATTTG

2101 CTCTAAACCAGATCTAAACCTGTATGTGTCGTATCCACATGTTTTGATAGTGACTGATTT

2161 TTTCCCTATTGCTGCACAATCCCAAGACATGTCATCTCCTTCTAGAGTTTTCTTAGTAAA

2221 AATAGGCACTCCATCATGGGTCAATTGTGGATGACCAAACATTTTCACAGGATCATTCAA

2281 GTTTGCAATATTTCCAGAGTAAATATGACTGTCGGGTCCATGAGCTATTAGTTCACCTAT

2341 AGTGATACCATCATTATGCAAATCTGCTTGTATATCAGCTTGAAACAATGTATTTTCATA

2401 AGGAACCTCTTCAGTAATCCTTGAGCATTGACCTCCCAAAATACCGGAAATACAAACATC

2461 TGCTACTATAGTTGACTTAAGCACTGAATAAATTCTGTATGATTTGTCCATATCATAAAT

2521 ATTTCGACAGAATCCGCATGTAGCACCCTCATTAATTGCAAAACACCAAGCTTCTTCACA

2581 TCCCCAATAAGAAGTTGGTGTTACACAAAAATCTTGGAAACCTGTTAAAGCTTGATTTTT

2641 CCTGCAAGTGTCGCAGTTTCCTGTACAAGTGGAATAAAAATCCGTGTGGGTGCTTTGGAT

2701 GGGAGCTGTCGTATATTTTTCTGACACTTCATAATGAATTCCCACACTTTTGATATAAAT

2761 CACAAATTTTTTGGCTGTTTCTGAGGTCTTGTCATTTAGCATGAATATAGTTCCTCCTCC

2821 TCCTAAAAGAGATTGTTCTATCATATATCTATATTTCCCGTCTACAACAGAATCAAAGAT

2881 TAATGATTGCCTGGGCAGAATGTTCTCTGGTATGCTTGTTTCGTTTTTGTTTAAGGAGTC

2941 TCCGGCAACCATTCCAGAAATTTTGCCCTGACGGTTATAGGAATCTATTATACCATTTCT

3001 CAATCTCTTAGCCTCATACAGACCAACTGATGTTAACCCTAAAGAGCTTCCTGTATAATC

3061 CGAAAACCCATTGTACAAAGGTTTGTTTCGGAATAAATCTAGGTATTCGCAACCTAATCT

3121 GCATTTTATAAGATTATCAATAGATGGGCGTGCAATGCAATTCTGGTTCTCTATGCAATC

3181 ATTAGGACCTTCTATAACAATATTAGTGCCAAAGATACTCTCTATGATCTTATCTTCTTT

3241 TACATTGCAGTAACATTGATCTTTTTCAGAGCATTTTTCAAATTTGCTTGTAACCAAAAA

3301 TGGACAGCCTGGAACATAAAAGCATCCACTCAAACATTGGGTTGTTTGAGCCATAGACAT

3361 GGGCATCTGAGACAAAATGACTAAACCTATCAAAATTTCGGTCACAAATTTTAGCAAACT

3421 CAAGCTCAGCTTAGTGTTCACTATTAGATGGAACCATTCCATGCTAGTCCACTTATGTTT

3481 GTTGTAGTCATGATCTGCTTCTTTGGATAGTATGGGACACTCTGAAGAATGCTCTTTTGA

3541 AGCTTTGCTTTTGTTGCAAATGCAGACTTTAGTACACTCATGTGTGACTATGCACAGATT

3601 GCCGCAGTTAGAACATTTTAATGGGAAATATTTCCATAAGCAATTTATGAGCAATAAGAC

3661 AGGGTATGTAATCAAGCCCATAAGATCATACCAGAGAAAGAGAGGTTTAGTCGTCTTGTT

3721 CACTAACCATCGGATAGGGAAATAGATCAACAAAGCTATCAATATCAACCTTATCCAAGA

3781 AAAATTGATGCAGGCTGTTTGCTTATAAATACTTTTTGAATATTTGATTATGCAATCTCT

3841 GACTCTTTTGTTTGTTTTTGGTATTTTGGCTGATTTGTCACCACACAAGAGATTGTGTTC

3901 ACCATCCAACATTTCCTCAGTAAAAGTGATACTTGCTGATCCAGAAAAAGATATAACCTT

3961 GTGTTCCACATTTTCACCAGGTTTTTTTATCAAATAACCCATGATCTTCTCAGGGCTAGT

4021 GATGCTTACAGTATAGGGATTTGCGAAGTTTGATTTAGTTATTTTGCAGTCACCGGATAA

4081 CTTTACAGTTTGTAATGATACTGTTCCATTAGTGGGGTATGAGTTGTAAGTTATAGGATA

4141 ATTATCTTGTGTCAGGCTTTCTGAAATGAAGAATTTTCCTCCTACTGAAAAGTGTCTTTT

4201 CTTGTCTAGCTTGGTAATGGGAATAACCGGGACTTTGGAGAATCTCTTTGGCAAATTTAA

4261 AGAATTATCACATTTTTCTAAACCTTCTGCTGAATCAGAAACACAGGAATATATGACACC

4321 ATTGTTTTCAACTTGATAATAAACATTATAAGTGGATATCCCTTTTATCTCACATTTTAA

4381 TGAAGAAGCATTCAAGCAATTGTTGGGAAGATCCAAAACAGAGATTGTTTTTTGCGTTGT

4441 TGGCTCAGCAGAAATAGGGATGGTTGATTTTTCTTCTCGTATCTGACGGGTTCCAGGAGT

4501 CCGAGATTCTAGCATCAGATTAGTTAAAGTCTCTAAGATAGCTTCGCGTTGAATCGATGC

4561 AGCAGTGGGTACCTCATTCTCAGCAGAATCATCATAAATCTCAGGATGATCTCCACGAAT

4621 TATTTCTACTTTAGCATCTGTGGCTCTGAAGATCAAGAATGCCAACAAAACAGAACTCAG

4681 GGCAATTGTGAAAAGACTCACTTTTACCACTAGTTCTAGTAGTTTTAGAATTCTCATCTT

4741 AGATGTCTACCCAGATTACAATGGTTGTGTGATTAATTTCAAGATGTCTGGATTAAGGTT

4801 TTTGTTTGCACTGATTGCTC

3. NUCLEOTIDE COMPOSITION OF LK-1 SMALL SEGMENT

1 AGAGCAATTGTGTCAGAATTTTGTTCATAATCAAACCTCACTTAGAAAATCACAATA
61 CTGTAATAAGAACACAGTACCAATAACCATAATGTCTTCAAGTGTTTATGAGTCGAT
121 CATTCAGACAAGAGCTTCAGTCTGGGGATCAACTGCATCTGGTAAAGCTGTTGTAGA
181 TTCTTACTGGATTCATGAACTTGGTACTGGTTCTCAACTAGTTCAGACCCAGCTGTA
241 TTCTGATTCAAGAAGCAAAGTAGTCCTTTGGCTATACTGCAAAGTAGGGATCTTCCC
301 TGTGAAGAAGAAGAGATTTCTTTCTCAGCATGTGTATATCCCTATTTTTGATGATAT
361 TGATTTTAGCATCAATATTGATAACTCTGTTCTGGCACTATCTGTTTGCTCAAATAC
421 AGTCAATGCTAACGGAGTGAAACATCAAGGTCATTTGAAGGTTTTGTCTCCTGCCCA
481 GCTCCACTCTATTGAATCTATCATGAACAGATCTGATATTACAGACCGATTCCAGCT
541 CCAAGAAAAAGACATAATTCCCAATGACAAATACATTGAAGCTGCAAACAAAGGCTC
601 TTTGTCTTGTGTCAAAGAGCATACCTATAAGATCGAGATGTGCTATAATCAGGCTTT
661 AGGCAAAGTGAATGTTCTATCTCCTAACAGAAATGTCCATGAATGGCTGTACAGTTT
721 CAAGCCAAATTTCAATCAAGTTGAAAGCAACAACAGAACTGTAAATTCTCTTGCAGT
781 GAAATCTCTGCTCATGTCAGCAGAAAACAACATCATGCCTAACTCTCAAGCTTCCAC
841 TGATTCTCATTTCAAGCTGAGCCTCTGGCTAAGGGTTCCAAAGGTTTTGAAGCAGGT
901 TTCCATTCAGAAATTGTTCAAGGTTGCAGGAGATGAAACAAACAAAACATTTTATTT
961 ATCTATTGCCTGCATTCCAAACCATAACAGTGTTGAGACAGCTTTAAACATTACTGT
1021 TATTTGCAAGCATCAGCTCCCAATTCGCAAATGCAAAGCTCCTTTTGAATTATCAAT
1081 GATGTTTTCTGATTTAAAGGAGCCTTACAACATTGTTCATGACCCTTCATACCCCAA
1141 AGGATCGGTTCCAATGCTCTGGCTCGAAACTCACACATCTTTGCACAAGTTCTTTGC
1201 AACTAACTTGCAAGAAGATGTAATCATCTACACTTTGAACAACCTTGAGCTAACTCC
1261 TGGAAAGTTAGATTTAGGTGAAAGAACCTTGAATTACAGTGAAGATGCCTACAAAAG
1321 GAAATATTTCCTTTCAAAAACACTTGAATGTCTTCCATCTAACACACAAACTATGTC
1381 TTACTTAGACAGCATCCAAATCCCTTCATGGAAGATAGACTTTGCCAGAGGAGAAAT
1441 TAAAATTTCTCCACAATCTATTTCAGTTGCAAAATCTTTGTTAAAGCTTGATTTAAG
1501 CGGGATCAAAAAGAAAGAATCTAAGGTTAAGGAAGCGTATGCTTCAGGATCAAAATA
1561 ATCTTGCTTTGTCCAGCTTTTTCTAATTATGTTATGTTTATTTTCTTTCTTTACTTA
1621 TAATTATTTCTCTGTTTGTCATCTCTTTCAAATTCCTCCTGTCTAGTAGAAACCATA
1681 AAAACAAAAAATAAAAATGAAAATAAAATTAAAATAAAATAAAATCAAAAAATGAAA
1741 TAAAAACAACAAAAAATTAAAAAACGAAAAACCAAAAAGACCCGAAAGGGACCAATT
1801 TGGCCAAATTTGGGTTTTGTTTTTGTTTTTTGTTTTTTGTTTTTTATTTTTTATTTT
1861 ATTTTTATTTTATTTTATTTTTATTTTATTTTTATTTTATTTATTTTTTGTTTTCGT

1921 TGTTTTTGTTATTTTATTATTTATTAAGCACAACACACAGAAAGCAAACTTTAATTA

1981 AACACACTTATTTAAAATTTAACACACTAAGCAAGCACAAGCAATAAAGATAAAGAA

2041 AGCTTTATATATTTATAGGCTTTTTTATAATTTAACTTACAGCTGCTTTCAAGCAAG

2101 TTCTGCGAGTTTTGCCTGCTTTTTAACCCCGAACATTTCATAGAACTTGTTAAGAGT

2161 TTCACTGTAATGTTCCATAGCAACACTCCCTTTAGCATTAGGATTGCTGGAGCTAAG

2221 TATAGCAGCATACTCTTTCCCCTTCTTCACCTGATCTTCATTCATTTCAAATGCTTT

2281 GCTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCCTTGGTGTCATACTTCTTTGG

2341 GTCGATCCCGAGGTCCTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGAT

2401 CATCTCAAAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGC

2461 AAGTCTCACAGACTTTGCATCATCGAGAGGTAATCCATAGGCTTGAATCAAAGGATG

2521 GGAAGCAATCTTAGATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCTTCAAC

2581 AAGCCTGACCCTGATCAAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGCCTCCAAT

2641 CCTGTCTGAAGTTTTCTTTATGGTAATTTTACCAAAAGTAAAATCGCTTTGCTTAAT

2701 AACCTTCATTATGCTCTGACGATTCTTTAGGAATGTCAGACATGAAATAACGCTCAT

2761 CTTCTTGATCTGGTCGATGTTTTCCAGACAAAAAGTCTTGAAGTTGAATGCTACCAG

2821 ATTCTGATCTTCCTCAAACTCAAGGTCTTTGCCTTGTGTCAACAAAGCAACAATGCT

2881 TTCCTTAGTGAGCTTAACCTTAGACATGATGATCGTAAAAGTTGTTATATGCTTTG

APPENDIX TWO
AGAROSE GELS

1. *TOMATO SPOTTED WILT VIRUS* (TSWV)

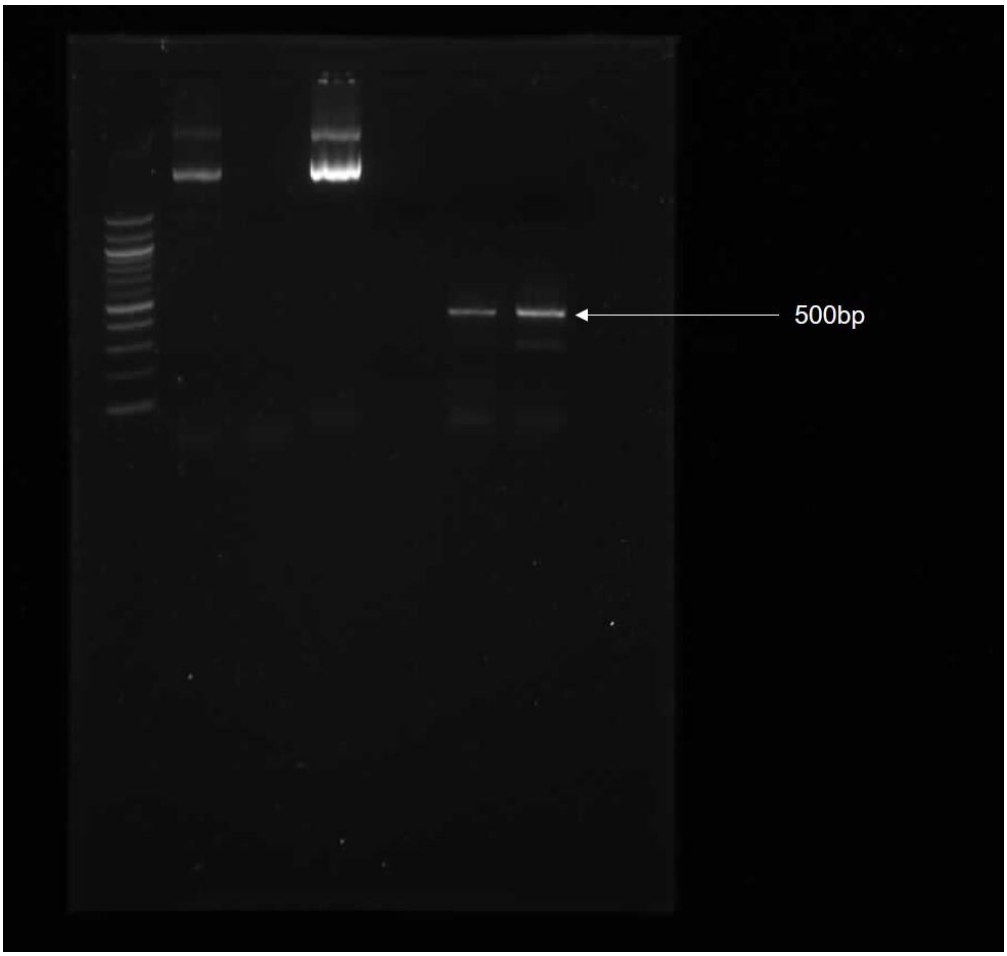

Figure 1: Agarose gel showing RT-PCR products using a *Tospovirus* primer.

2. *TOMATO TORRADO VIRUS* (ToTV)

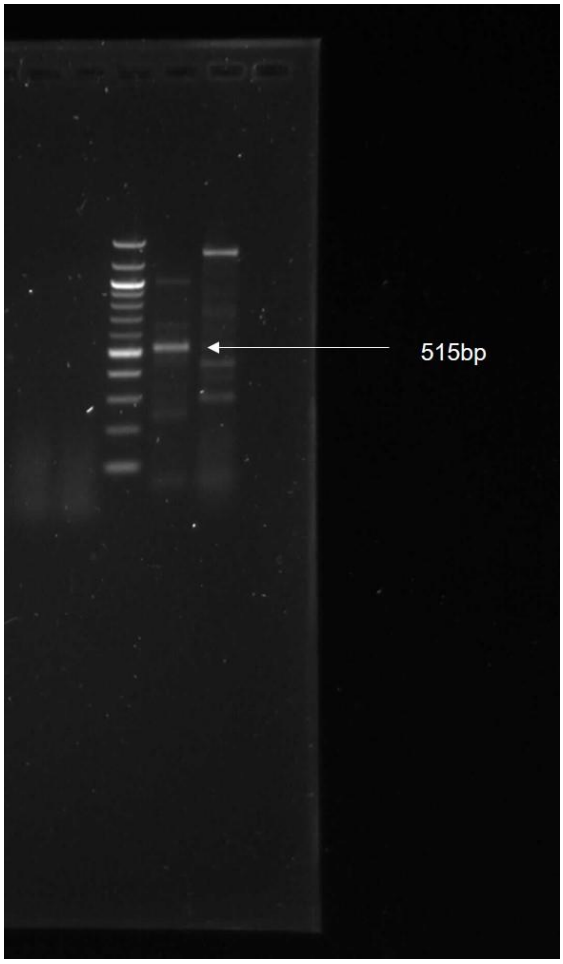

Figure 2: Agarose gel showing RT-PCR products using a *Torradovirus* primer.

3. *TOMATO CHLOROSIS VIRUS* (ToCV) AND *POTATO VIRUS Y* (PVY)

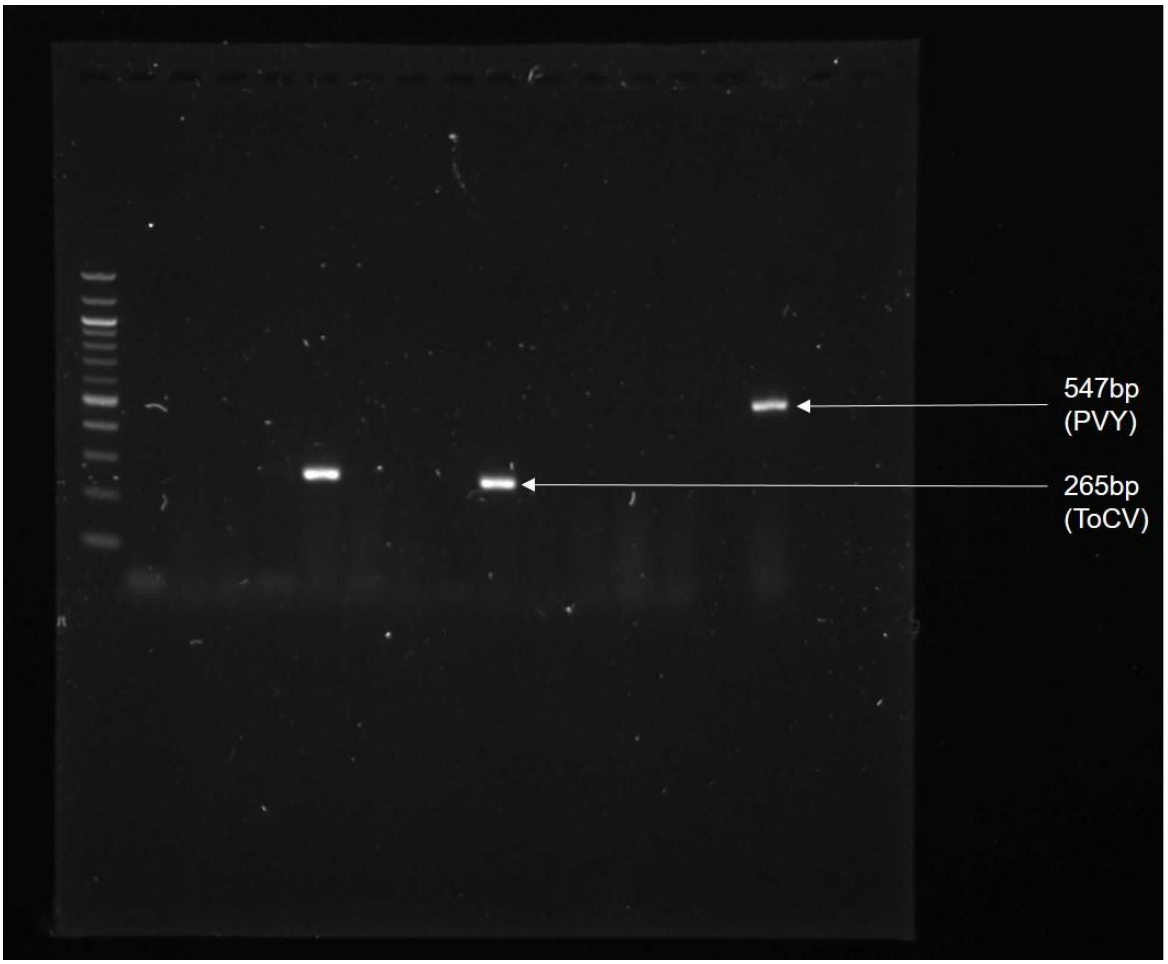

Figure 3: Agarose gel showing RT-PCR products using a *Crinivirus* and PVY-specific primer.