OCCURRENCE, CHARACTERISATION AND DISTRIBUTION OF VIRUSES INFECTING TREE TOMATO (*Solanum betaceum* CAV) IN KENYA

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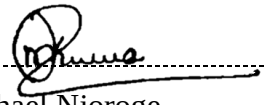
Signature  Date 26th May, 2023

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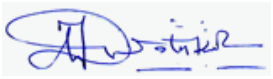
Signature  Date 31/05/2023

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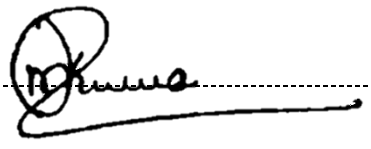
Signature  Date 26th May, 2023

DEDICATION

To my wife, parents, brothers and their families who, because of their patience, advice and prayers, have been the motivation to complete my masters. To the Immaculate heart of Mary, whose intercession has encouraged me to grasp on to the grace of God when things became difficult. To the almighty God, whose Eucharistic presence nourished me, and strengthened me at every moment.

Sweet sacrament divine
Hid in thine earthly home
Lo, round thy lowly shrine
With suppliant hearts we come
Jesus, to thee our voice we raise
In songs of love and heartfelt praise
Sweet sacrament divine
Sweet sacrament divine

Sweet sacrament of peace
Dear home of every heart
Where restless yearnings cease
And sorrows all depart
There in thine ear all trustfully
We tell our tale of misery
Sweet sacrament of peace
Sweet sacrament of peace

Sweet sacrament of rest
Ark from the ocean's roar
Within thy shelter blest
Soon may we reach the shore
Save us, for still the tempest raves
Save, lest we sink beneath the waves
Sweet sacrament of rest
Sweet sacrament of rest

Sweet sacrament divine
Earth's light and jubilee
In thy far depths doth shine
The Godhead's majesty
Sweet light, so shine on us, we pray
That earthly joys may fade away
Sweet sacrament divine
Sweet sacrament divine

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ABBREVIATIONS AND ACRONYMS

µl	Micro litre
µM	Micro Molar
AMV	Alfalfa mosaic virus
ArMV	Arabis mosaic nepovirus
BLAST	Basic local alignment search tool
Bp	Base Pair
CMV	Cucumber mosaic virus
CP	Coat protein
CTAB	CetylTrimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
ETBTV	Ethiopian tobacco bushy top virus
HCl	Hydrochloric Acid
HCPro	Helper component proteinase
HSD	Honest significant difference
LAMP	Loop-mediated isothermal amplification
LH	Lower highland
LSD	Least significant difference
MEGA	Molecular evolutionary genetics analysis
ML	Midland
MT	Metric tonnes
NaCl	Sodium Chloride
NGS	Next generation sequencing
NP	Nucleoprotein
PAMV	Potato Aucuba mosaic virus
PCR	Polymerase Chain Reaction
pH	Potential Hydrogen
PLRV	Potato leafroll virus
PSTVd	Potato spindle tuber viroid
PTV	Peru tomato mosaic virus
PVP	Polyvinylpyrrolidone
PVS	Potato virus S
PVV	Potato virus V
PVX	Potato Virus X
PVY	Potato virus Y
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic Acid
RT	Reverse transcriptase
satRNA-E	Ethiopian tobacco bushy top virus RNA satellite
TaLMV	Tamarillo leaf malformation virus
TamMV	Tamarillo mosaic virus
TAV	Tomato aspermy virus
TBE	Tris Boric Acid
TE	Tris EDTA
TMoV	Tobacco mottle virus
ToCV	Tomato chlorosis virus

TMMoV	Tomato mild mottle virus
ToMV	Tomato mosaic virus
ToRSV	Tomato ringspot virus
Tris	Tris (Hydroxymethyl) Aminomethane
TSV	Tobacco streak virus
TSWV	Tomato spotted wilt virus
UH	Upper highlands

ABSTRACT

The tree tomato is a shrub belonging to the *Solanaceae* plant family that bears egg-shaped berries. Main production constraints include diseases, pests and drought. Diseases caused by fungi, bacteria and viruses are the largest contributor to farmer's losses. Viruses are known to cause the heaviest economic losses. In Kenya, only two viruses, potato virus Y (PVY) and tomato mild mottle virus (TMMoV), have been previously detected infecting the crop. This study was conducted to identify and characterize the identified viruses infecting the crop, and determine their prevalence and distribution in the country.

Two surveys were conducted in three agro-ecological zones (upper highlands, lower highland, and midlands) in the main tree tomato production counties of Machakos, Nairobi, Embu, Tharaka Nithi, Meru, Elgeyo Marakwet, Nandi, Baringo and Nakuru between 2018 and 2019. During the survey, 26 farms were visited and 358 tree tomato leaf samples showing symptoms of virus infection such as mosaics, vein clearing, vein banding, and leaf malformation were collected. It was observed that the severity of these symptoms was more pronounced in the midland and lower highland zones.

Using next generation sequencing (NGS) three viruses, an RNA satellite, and a viroid were identified: potato virus Y (PVY), tomato mild mottle virus (TMMoV, 9243 bp, MW537585), Ethiopian tobacco bushy top virus (4199 bp, MW883068), and associated RNA satellite (522 bp, MW713579), and lastly potato spindle tuber viroid (359 bp, MZ054164). The PVY genome was not established. This is because the reads were from pooled sample, and plus the fact that the similarities in the recombination pattern and recombinant breakpoints among the genomes of recombinant strains would have led to the formation of a chimeric genome that does not exist.

Results from NGS for all 3 viruses and the viroid were confirmed by RT-PCR using virus specific primers and Sanger sequencing. By pair-wise alignment, the sequences obtained from Sanger sequencing were all aligned to their respective NGS derived genomes and showed: 90% for ETBTV, 99% for satETBTV-E, 96% for TMMoV and 98 for PSTVd sequence identity.

The PCR diagnostics were then used to determine the incidence and prevalence of each of the viruses. PVY was the most prevalent virus with 46% of all farms having samples which tested positive. Apart from Elgeyo Marakwet, all other counties had farms with samples had tested positive for PVY. Both TMMoV and PSTVd were present in two farms (from Embu and Meru), while ETBTV and its RNA satellite were present in one farm (from Tharaka Nithi). This is the first report of ETBTV and its RNA satellite infecting tree tomato, as well as the first report of a natural infection by PSTVd. Other viruses (pepper enomavirus, tobacco mottle virus, tomato chlorosis virus, Kenyan potato cytohabdovirus and tamarillo fruit ring virus) were also detected using NGS, however research is still ongoing in KALRO to characterise and validate their presence using RT-PCR.

The results of this study have expanded the knowledge of the number of viruses infecting tree tomato in Kenya and developed primers and RT-PCR assays for quick diagnosis thereby allowing for consistent detection of viruses within commercial nurseries and farmers' fields consistent with virus infection. The ability to perform routine diagnosis of these viruses is an essential tool for seed certification programs, breeding programmes, epidemiological monitoring and evaluation studies, and for development of management strategies.

CHAPTER 1 INTRODUCTION

1.1 Tree tomato industry

Tree tomato or tamarillo (*Solanum betaceum* Cav.) is a member of the *Solanaceae* family native to the Andean region of South America (Morton, 1982). It is considered a sub-tropical plant mostly grown in the highland areas. It is grown for its edible fruits, which can be consumed directly and/or processed into industrial products such as jams and juice. It is high in minerals, vitamin A and C content, and low calorie, qualities that have made it to be highly sought after by health diet conscious consumers (Prohens and Nuez, 2001). The fruit extracts have been found to reduce cholesterol levels in cases of obesity (Abdul-Kadir *et al*., 2015) as well as chemo-preventive and anticancer properties against breast and liver cancer cells (Abdul-Mutalib *et al*., 2017).

In Kenya, the tree tomato industry is still in its infancy, and as at 2017, tree tomato only contributed less than 1% of the total value of fruits produced (HCD, 2017). It has however been gaining popularity in urban areas where it is mostly eaten raw or used for juice or in food processing industry (Muriithi *et al*., 2013; HCD, 2017). Because of the increasing popularity, production has been increasing and with proper promotion, it has a great potential as an economic enterprise for many farmers. It however faces a number of hurdles, the main constraint being diseases, followed by pests and bad weather (Muriithi *et al*., 2013). In the recent past, it has received little attention from research with very little data published on the main diseases constraining production in Kenya. In other countries, it has been found to be susceptible to several pathogens such as fungi, viruses, bacteria, insects and nematodes (Prohens and Nuez, 2001). Of these pathogens, viruses have been found to be the most important phytosanitary problem that limit production (Jaramillo *et al*., 2011; Mejía *et al*., 2009). This is quite significant, in our context especially considering the fact that tree tomato contributes almost 1.2 billion Kenya shillings to the horticultural sector (HCD, 2020).

1.2 Statement of the problem

Virus infections have been found to cause heaviest losses in tree tomato in other countries such as Colombia and New Zealand (Mejía *et al*., 2009). In Colombia for example, in an area called Antioquia, viruses were attributed to losses of about 50-80% of cultivated area (Jaramillo *et al*., 2011; Mejía *et al*., 2009). The viruses affect the health and vigour of the crop but also damage the skin of the fruit causing it to lose aesthetic value especially for the export market (Atkinson and Gardner, 1993).

Tree tomato has been identified to be susceptible to about 20 viruses in other countries, which occur in either single or mixed infections (Eagles *et al*., 1994; Jaramillo *et al*., 2011). Of the 20, Tomato mosaic virus (ToMV), Potato aucuba mosaic virus (PAMV), and Arabis mosaic nepovirus (ArMV) have been found to infect other hosts in other African countries, while potato virus Y (PVY), tomato mild mottle virus (TMMoV), alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), potato leafroll virus (PLRV), tomato spotted wilt virus (TSWV), potato virus S (PVS), and potato virus X (PVX) have been detected in other hosts in Kenya (Kaiser, 1980; Ames, *et al*., 1996; Ssekyewa, 2006; Wangai and Lelgut, 2006; Were, *et al*., 2013; EFSA PLH Panel, 2013; Macharia, *et al*., 2015). Only PVY and TMMoV have been previously detected in tree tomato in Kenya (Mbula, 1992; Monger and Nixon, 2010), while the coat protein sequence of tomato mild mottle virus (TMMoV) has also been detected with a partial coat protein gene recorded in GenBank (Acc. No. HQ711860; Monger and Nixon, 2010). Although tree tomato production has been on the increase, area under production has been on the decrease in some areas. A good example is Kiambu, where, in 2016, area under production stood at 189 ha, but as of 2020 it had reduced to 71 ha (HCD, 2017; HCD, 2020). This represents a 62% reduction in a matter of four years in the county which had the highest production in 2016 (HCD, 2017; HCD, 2020). Meru also raises concern as it has the second largest area under cultivation at 212 ha yet it produces a mere 1073 MT of fruit as compared

to a county like Elgeyo Marakwet at 82 ha yet it produces three times the weight of fruits, at 3220 MT (HCD, 2020). This reduction in area under production in Kiambu county could be an indication of abandonment for more profitable ventures by farmers, while the lower yields in Meru county compared to Elgeyo Marakwet county could be as a result of diseases and other constraining factors which lower yields. There is currently no guideline, based on scientific research, for controlling tree tomato virus diseases in Kenya. This is a red-flag that the sector is in danger and that measures should be instituted to urgently stop the decline.

1.3 Justification

Control strategies of viruses rely on a particular dependency order of workflow of information: first is a thorough knowledge of the invading virus(es); second is knowledge of the source of initial inoculum; and thirdly the mode of transmission should be known (Stevens, 1983). Prevention and assuagement strategies of viral diseases are all dependent on this workflow. In Kenya, only PVY and TMMoV have been identified infecting tree tomato. Currently, there is no literature available which has identified other virus(es) infecting tree tomato in Kenya, and none providing epidemiological data such as incidence and severity, modes of spread, vector identity and population. The absence of this information has so far hindered sector players from coming up with mitigation measures such as type of pesticides to use and provision of clean seed to farmers. Lack of this information has been a discouraging factor to potential as well as to existing farmers from expanding production. This has led to most of the farmers to only manage production on small scale for subsistence (Muriithi *et al*., 2013). Considering that not much virus research has been done on the crop in the country, there is a likelihood that there are more viruses infecting the crop, and that some plants may be infected by a complex of viruses at any one point as seen in other tree tomato growing countries (Eagles *et al*., 1994; Jaramillo *et al*., 2011).

Since tree tomato farming is still in its infancy, it is paramount that certain prophylactic measures be taken to either counter the spread or prevent infection by eliminating known sources of inoculum, control vectors, and begin breeding for resistance against identified virus(es). This proactive approach will serve to offer guidelines for farmers venturing into tree tomato farming, and assist the concerned plant health inspectors in coming up with measures to prevent known quarantine pathogens from entering into the country to protect the local sector. The first step in this process is to identify the viruses infecting the crop in the country and determine their distribution, which is the focus of this study.

1.4 Objectives

1.4.1 Main objective

The main aim of this study is to contribute to sustainable production of tree tomato in Kenya through understanding the viruses infecting the crop and their distribution in different agro-ecological zones.

1.4.2 Specific objectives

i. To identify and characterize viruses infecting tree tomato in Kenya
ii. To determine the distribution of viruses infecting tree tomato in Kenya

1.5 Hypothesis

i. Tree tomato production in Kenya is affected by a wide diversity of viruses.
ii. Viruses infecting tree tomato are widely distributed in Kenya.

CHAPTER 2 LITERATURE REVIEW

2.1 Botanical description of tree tomato plant

Tree tomato is a fruit-bearing tree of the Solanaceae family. The height of tree tomato ranges between 3 – 5.5 m. The leaves are alternately arranged, the base is mostly cordiform at the base and a pointed apex. The flowers appear in clusters near the tips of branches, 1.25 – 2 cm in width, the corolla is pinkish-white with 5 lobes which are pointed, stamens are 5 and yellow in colour, and a purplish coloured calyx. They bear egg-shaped fruits pointed at the two ends, have a length of between 5 – 10 cm and 4 – 5 cm in width, the calyx is persistent and the skin has several colours ranging from red to yellow to purple while the flesh ranges from yellow to orange to red. The skin of the fruits may or may not have faint dark coloured stripes, (Figure 2.1; Morton, 1982).

2.2 Origin and distribution of tree tomato

Tree tomato is native to the Andean region of South America, mainly Peru and probably also Chile, Ecuador, Argentina and Bolivia (Prohens and Nuez, 2001; Morton, 1987). It is cultivated in several countries of the world, including: southern States of the United States, Mexico, most of South America, the New Zealand, a few other European countries, East Asian countries, the Indian sub-continent, Australia and its neighbouring Pacific Islands, and lastly in Africa it is grown in Egypt, Kenya, Uganda, Tanzania, and South Africa (Orwa *et al*., 2009). In Kenya tree tomato is largely produced in Nyandarua, Uasin, Gishu, Elgeyo, Marakwet, Meru, Kirinyaga, Kiambu, Narok, Nakuru, Murang'a, Tharaka, Nithi, Nyeri, Laikipia, Baringo, Nandi, and Makueni county (HCD, 2020).

Figure 2.1 A tree tomato plant and red fruit shown on the bottom left corner *(courtesy of floridaseeds.net)*

2.3 Optimum climatic conditions for tree tomato production

Tree tomato is considered to be a sub-tropical plant (Morton, 1987). It generally grows well in well drained soils at altitudes of between 1000 m to about 3,000 m above sea level, with temperatures of between 15 – 22 ºC and an annual rainfall of between 1000 – 1600 mm (Orwa *et al*., 2009). Kenya lies within the tropics and its climate is ideal for tree tomato cultivation. It is grown in the Rift Valley and around the Mount Kenya region which have a mean annual total rainfall of between 500 – 1800 mm, temperature ranges between 9 – 31°C, an altitude of between 300 and 1700 m (Kilavi, 2012) and soils which are well drained and of volcanic origin (FAO, 2005).

2.4 Cultivar groups of tree tomato

Tree tomato cultivar groups, referred as such because they are not true cultivars (Cornejo-Franco *et al*., 2019), are yellow, red, and purple. Yellow are characterised by having yellow skin with brown-green stripes and yellow flesh. Red have a red skin with green-brown and orange flesh stripes. Purple have a dark-red skin with light green stripes and purple flesh. Yellow and red fruits weigh roughly between 50 – 80 grams. Purple varieties have heavier fruits ranging from 60 to100 grams (Prohens and Nuez, 2001). Red and purple varieties are a favourite in American and European export markets because of their acidic taste, while the yellow varieties are preferred for canning as the lower acid content means they are less likely to corrode cans (Wratt and Smith, 1983). Breeding programs in New Zealand and California have produced several cultivars based on these fruit colour: yellow fruiting includes, egmont gold, inca gold and goldmine; red fruiting includes, oratia red, red delight, red beam, Ecuadorian orange, secombes red, andys sweet red, red beauand solid gold; and the dark-red or purple variety includes, kaitaia, holmes, ruby red and rothamer (Prohens and Nuez, 2001). In Kenya, there has been very few breeding efforts for tamarillo, and information about known

varieties grown in Kenya is still scarce. They include ruby red, inca red, red oratia, gold mine, solid gold and, rothamer (HCD, 2017).

2.5 Economic importance of tree tomato in Kenya

Tree tomato is an emerging crop and is still highly underutilised (HCDA, 2008; Kanali *et al*., 2017). Between the years 2005 and 2007, it formed a mere 0.14% and 0.18% of the total value of fruits produced in these three years. In 2020, production had grown to about 1.35% of the total value of fruits, which amounted to 21,776 tons up from 10,089 tons in 2017, and covering 1,321 ha of land, and valued at about Kenya shilling 1.2 billion, with an average price of approximately KShs 50 per kg (HCD, 2018; HCD, 2020).

A study done in Embu county showed that most of the farmers growing tree tomato did it for household consumption, although there was growth in both commercial and subsistence farming (Muriithi *et al*., 2013). This growth seemed to be on an upward trend as more and more people became aware of tree tomato and its perceived health benefits to expectant mothers in increasing blood (Riang'a *et al*., 2017). There is however more potential yet to be exploited as farmers and other sector players embrace value addition to produce jams, juice, wine and other products where it can fetch better prices.

2.6 Pests and diseases of tree tomato

2.6.1 Insect pests

The main pest is the tree tomato worm, which is the larva of the moth, *Neoleucinodes elegantalis*, which bores into the fruit and causes it to rot because of secondary infections (Bioversity International, 2013). It can cause up to 80% fruit loss (Orwa *et al*., 2009). Tomato-potato psyllid is also a major pest of tree tomato whereby it transmits *Candidatus liberibacter solanacearum* which has impacted severely on the tree tomato industry in New Zealand

(Edwards, 2014). The leaf-footed bug feeds on the fruits causing them to harden in certain areas due to a toxin from their saliva and the appearance of dark spots on the fruits. It also acts as a *Colletotrichum gloeosporoides* vector which causes anthracnose (Quezada, 2011). Aphids is also an important pest which feeds on the veins on lower side of the leaf, and produces honeydew (Edwards, 2014) as excrement which then provides a substrate for growth of saprophytic fungi and as a result, cause reduced growth rate of shoots and other plant parts (Quezada, 2011). In addition to this, they also act as vectors for some viruses (HCD, 2018). The red spider mites have also been identified to be a major pest of tree tomato (HCD, 2017). The greenhouse whitefly (Bioversity International, 2013) feeds on the lower part of the leaf and excretes honeydew which acts as a substrate for growth of saprophytic fungi. It can cause considerable leaf loss, leading to reduced yield by up to 75% (Prohens and Nuez, 2001).

2.6.2 Nematodes

Root-knot disease is also a major problem which is caused by *Meloidogyne incognita, M. hapla* or *M. javanica* (Bioversity International, 2013). The disease is exhibited as large galls which distorts the roots inhibiting their ability to translocate nutrients and water (Sale, 1983). *Xiphinema diversicaudatum* also infests tree tomato and acts as a vector for Arabis mosaic nepovirus (ArMV) (Prohens and Nuez, 2001; Sale, 1983). A less significant nematode pest that also affects tree tomato is *Pratylenchus crenatus* (Bioversity International, 2013).

2.6.3 Fungal diseases

Anthracnose caused by *Colletotrichum acutatum* or *C. gloeosporioides*, is considered to be a very important fungal disease affecting tree tomato in several countries (Edwards, 2014), causing losses which can reach between 50 – 100% (Quezada, 2011). Symptoms appear as oily lesions on the fruits which turn black or brown with distinct edges and a pinkish centre which

indicates sporulation. As the disease progresses the lesions coalesce and mummify that part of the fruit. On the leaves the disease is manifested by the presence of spots with dark coloured concentric rings (Quezada, 2011).

Powdery mildew, caused by *Oidium* sp. or *Erysiphe* sp., is also an economically important disease. Symptoms appear as a dark spot on the leaves surrounded by a whitish powder (Quezada, 2011), indicating sporulation. As the disease progresses, the spots coalesce resulting in severe defoliation (Morton, 1982).

Phytophthora infestans also causes significant losses in tree tomato. Symptoms appear on the leaves as wet spots, brown or black in colour, with a whitish powdery substance on top. Pedicels are also affected when defoliation occurs, and also fall resulting in reduced yields (Quezada, 2011). *Phytophthora cryptogea* causes root rot which is manifested by wilting, tree death and at times production of small fruits (Sale, 1983).

Sclerotiniosis or Sclerotinia disease, caused by *Sclerotinia sclerotiorum,* manifests symptoms on the trunk as dry light brown lesions with a soft white surface which later form hard black fruiting bodies called sclerotes either inside or outside the stem (Sale, 1983). The disease also results in defoliation (Quezada, 2011).

Other fungal diseases which affect tree tomato include: tamarillo leaf spot caused by *Phoma exigua* which manifests as brown coloured lesions on older leaves (Bioversity International, 2013); *Alternaria* sp. causes fruit rot and early blight; *Fusarium solani* causes stem black lesion; and *Ascochyta* sp. causing ascochyta disease (Morton, 1982).

2.6.4 Bacterial diseases

Pseudomonas syringae causes bacterial blast in tree tomato and shows different symptoms depending on the fruit colour (Sale, 1983). On cultivars with red fruits, symptoms appear as

light brown or water-soaked lesions which later become dark brown and fall off creating shot holes on the leaves. Bacterial infection of the fruit and flower stalks is manifested by the appearance of a black wet lesions going around the stalk which later causes the fruit to drop (Sale, 1983; Edwards, 2014). In cases where tree tomatoes are grown close to tomatoes, *Corynebacterium michiganense* may be found causing cankers on the fruits (Sale, 1983) of tree tomato. Infection by *Candidatus liberibacter solanacearum,* is spread by the tomato-potato psyllid, results in stunted growth, chlorosis and branch dieback which results in tree death (SPHDS, 2012).

2.6.5 Viral pathogens

Several viruses have been identified to infect tree tomato, so far they number 20 and include alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), tomato ringspot virus (ToRSV), potato leafroll virus (PLRV), PVY, potato virus A isolate TamMV (PVA), tomato mosaic virus (ToMV), TMMoV (Monger and Nixon, 2010), tomato spotted wilt virus (TSWV) (Jaramillo *et al*., 2011), potato Aucuba mosaic virus (PAMV), Arabis mosaic nepovirus (ArMV), (Eagles *et al*., 1994), potato virus S (PVS, Ochoa and Tolin, 2012), potato virus V (PVV), Peru tomato mosaic virus (PTV) (Insuasti *et al*., 2016), potato virus X (PVX), tobacco streak virus (TSV), tomato aspermy virus (TAV) (Quezada, 2011), tamarillo leaf malformation virus (TaLMV) (Gutierrez *et al*., 2015), broad bean wilt fabavirus (BBWY) (Pliansinchai and Teakle, 1994), and naranjilla mild mosaic virus (NarMMV) (Cornejo-Franco *et al*., 2019). These viruses have been known to cause disease as single virus infections or as complexes.

Only PVY and TMMoV have been detected infecting tree tomato in Kenya (Mbula, 1992; Monger and Nixon, 2010). Seven of the viruses just mentioned, have also been detected in Kenya infecting plants in the nightshade family. These viruses include: PVX, PLRV (Wangai and Lelgut, 2006), TSWV (Wangai *et al*., 2001), CMV (Opiyo *et al*., 2010), PVS (Were *et al*.,

2013), AMV (Kaiser, 1980) and TMV (Otieno, 1985). Those which have not been detected in Kenya so far but are present in other African countries include ToMV (Ssekyewa, 2006), PAMV (Wangai and Lelgut, 2006), ArMV (EFSA PLH Panel, 2013).

2.7 Detection methods for viruses infecting tree tomato

Common methods used in detection of viruses are polymerase chain reaction (PCR) based and enzyme linked immunosorbent assay (ELISA; Baranwal *et al*., 2020). Other methods which have been used are electron microscopy and dot blot analysis. Recently PCR and DNA hybridisation methods have however been found to be much more sensitive, in terms of the titre threshold, whereby they are able to detect low virus concentrations and are less affected by impurities as compared to serological methods like ELISA (Griesbach, 1995), and thus are much more preferred. PCR is the most user-friendly detection method targeting nucleic acids. In order to develop a PCR protocol for the detection of a particular virus, specific information about its nucleic acid sequence is required. This information is generated by sequencing of the viral genome and development of specific primers which can hybridize with specific target sites of the viral genetic material. Since the size of the target site is known, it allows for the particular virus to be identified with ease. Like PCR, Loop-mediated isothermal amplification (LAMP) is also a nucleic acid-based detection method. Although discovered over 20 years ago by Notomi *et al.* (2000), it is yet to be applied to tree tomato virus research.

In 1977, two methods ushered in the era of sequencing namely: The Sanger or dideoxy chain termination method which later became the model for all sequencing methods and received more adoption by the scientific community even to be used in the human genome project; and the Maxam and Gilbert or chemical sequencing method (Amaro *et al*., 2016). After the human genome project ended, scientists found that there was need to make whole genome sequencing faster. This was done by coming up with ways where sequence information could be obtained

in a parallelized fashion. Such methods came to be known as next generation sequencing (NGS). NGS has so far allowed whole sequences to be done within very short periods of time compared to the time it took for the human genome project to be completed. Viruses have much smaller genomes and it takes even a shorter time to sequence and analyse their genomes. NGS is very important for discovery of new viruses, and in phylogenetic studies. Its results are very important in designing of assays such as PCR which are much cheaper and can be used regularly in simple diagnostic.

Using cutting edge technologies like NGS, goes a long way in identifying more viruses infecting tree tomato which is highly understudied. Previously physical properties – dilution end point, thermal inactivation point and longevity *in vitro* – have been used to identify and characterize PVY in tree tomato in Kenya (Mbula, 1992). Today, these methods are considered highly unreliable in characterization, identification and classification of viruses (Francki, 1980). Apart from the use of physical properties, there is no record available of any of the more reliable diagnostic methods being used in Kenya in the diagnosis of viruses in tree.

CHAPTER 3 MATERIALS AND METHODS

3.1 Identification and characterization of viruses infecting tree tomato in Kenya

3.1.1 Sample collection and measurement of virus associated disease symptom

Surveys were carried out in tree tomato growing regions, in three agro-ecological zones in Machakos, Embu, Tharaka Nithi, Meru, Elgeyo Marakwet, Nandi, Nakuru, Baringo and Nairobi counties between December 2018 and August 2019. Selection of orchards was done based on information gathered from Agricultural extension officers, and a 5 km radius was kept between each orchard. The location (latitude and longitude) and altitude of each orchard was recorded using a global positioning system (GPS) device. Based on the recorded altitude of each site, each orchard was later categorised as falling into one of three agro-ecological zones, midland (1500 – 1850m), lower highland (1850 – 2450m) and upper highland (2450 – 3050m), as determined by the Kenya soil survey (1980; Appendix I and II). In each farm, famers were presented with a questionnaire, asking for: details of the famer, such as name, gender, age; details of the farm such as size and number, number of trees, size of harvest, income from harvest; and details of the agronomic practices implemented in the farm such as, fertilizer application, pesticide and herbicide application and disease management strategies (Appendix III). Trees in the orchards were examined from a random starting point and proceeding along an M-shaped path.

Disease severity for each orchard, was determined using an ordinal rating scale, adapted from Murphy (2000), based on common virus associated symptoms: mosaic, mottling, vein clearing and banding, yellowing, stunted growth and necrosis which were observed on the plants examined (Table 3.1). Plants showing symptoms on leaves, fruits and stems, consistent with virus infection were counted and compared as a percentage of the total number of plants examined to determine disease incidence. Disease prevalence was determined as a ratio of

fields showing symptoms in each county while incidence was determined by the ratio of number of plants showing symptoms.

Table 3.1 Disease severity scale used for rating of virus diseases infecting tree tomato during a survey in Kenya

Rating	Description
1	Symptomless
2	Mild mosaic, mottling, yellowing on young leaves
3	Obvious mosaic, mottling, vein clearing and banding, yellowing on leaves from at least one of the main stems
4	Obvious mosaic, mottling, vein clearing and banding, yellowing on leaves over of the entire plant
5	Obvious mosaic, mottling, vein clearing and banding, yellowing, leaf malformation, and severe stunting

Leaf samples were picked from plants showing symptoms, pressed in paper towels and placed in zip-lock bags containing silica gel (a desiccant) and transported to the laboratory for virus assays and identification. In the laboratory, the dry desiccated leaf samples were arranged in ascending order of the sample's codes used during sample collection. All leaves collected from one tree constituted one sample. A note on the insect pests, potential virus vectors was made during the survey.

3.1.1.1 Statistical analysis of disease symptom prevalence, incidence, and severity

Disease incidence and severity data were subjected to one-way analysis of variance (ANOVA) using excel to test for significant differences. Mean comparisons of the disease symptom incidence and severity was done using ANOVA at 95% confidence level. Tukey-Kramer post hoc was used for pairwise comparison of significant differences in prevalence, incidence, and severity means.

3.1.2 Virus identification

3.1.2.1 RNA extraction and cDNA Synthesis

Total RNA was extracted from the collected leaf samples using cetyltrimethylammonium bromide (CTAB) protocol as described by Gambino (2008) with modifications. Approximately 1 cm^2 piece of dry leaf tissue was obtained from each sample, ground using a motor and pestle, and mixed with 1 ml of preheated extraction buffer (2% CTAB, 5% PVP-40, 2 M NaCl, 100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0 and 2% of β-mercaptoethanol). This was followed by incubation at 65°C for 30 minutes and spinning at 13,000 rpm for 1 minute. An 800 µl volume of the supernatant was transferred to a 2 ml microcentrifuge tube and an equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added. The tube was gently inverted several times, and then centrifuged at 13,000 rpm for 10 minutes. After centrifugation, 800 µl of the supernatant was transferred to a new microcentrifuge tube with 800 µl of 5M LiCl, then incubated at -20°C for 1 hour. RNA was selectively pelleted after centrifugation at 13,000 rpm for 20 minutes. The supernatant was decanted and the pellet re-suspended in 200 µl of TE buffer (10mM Tris–HCl pH 8, 1 mM EDTA pH 8) then incubated at 65°C for 20 minutes. After centrifugation, 100 µl of NaCl was added followed by 300 µl of ice-cold isopropanol. The mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was decanted and the pellet washed with 400 µl of 70% ethanol and then centrifuged at 13,000 rpm for 5 minutes. The ethanol was decanted and the pellet air dried for 15 minutes and then re-suspended in 50µl of RNase free water. The RNA was allowed to reconstitute for 10 minutes at room temperature. The concentration of the RNA pool was measured using a Nanodrop spectrophotometer and diluted down to 2 µg/µl. Five (5) µl of the RNA from one randomly selected representative sample from every farm in Machakos, Embu, Tharaka Nithi, and Meru counties were picked and used to make a pool to be taken for sequencing. The half of the pool and the rest of the

RNA was preserved in a -80ºC freezer. The rest of the pool was taken for cDNA synthesis and next generation sequencing.

3.1.2.2 cDNA library preparation and next generation sequencing

Reverse transcription using RevertAid First Strand cDNA Synthesis Kit™ (Thermo Scientific™) was done using random hexamer primers according to manufacturer's protocol. A 50 µl reaction was prepared as follows: 10 µl of the RNA was mixed with 2.5 µl of random hexamer primers, 5 µl of 10 mM dNTP, 10 µl of the reaction buffer, 2.5 µl of the RNase inhibitor, 2.5 µl of the reverse transcriptase enzyme and 17.5 µl of nuclease-free water. The contents were incubated first for 5 minutes at 25°C, then the reaction was done for 60 minutes at 42°C. Quantification of cDNA was done using a Qubit® Fluorometer (Thermo Scientific™). Half of the cDNA was taken to KALRO marker assisted animal laboratory where they were sequenced on the Ion S5 XL system using an Ion 540™ Chip Kit (Thermo Scientific), and the other half kept at –20°C for later use in primer optimisation and source of positive controls.

3.1.2.3 Genome assembly, characterization and sequence analysis

For sequence data, sequencing reads were uploaded to CLC Genomics workbench (QIAGEN Aarhus, Denmark), and quality control done by trimming adapter sequences and low-quality bases from the raw reads using default parameters (reads with inaccurate consensus sequences nucleotide base calling, as well as those with low quality scores as determined by the sequencer). All reads having less than 50 bp length were also removed. The reads were compared to a local download of the GenBank virus database using basic local alignment search tool (BLAST+). In order of hierarchy of importance, identification of potential viruses was done for those which had: high read sequence coverage; high percentage identity; and high virus nucleotide sequence covered by contigs. The accessions of individual reads identified to

be aligning to specific viruses, were extracted from the contigs multi-fasta file to a smaller file. The reads aligning to the different viruses, were used to assemble virus genomes using a reference guided genome alignment tool in CLC Genomics workbench, with the respective virus sequences, from NCBI RefSeq genomes, as the reference sequence. Annotation of the protein coding sequences of the new genomes was done by comparing and extrapolation of the annotation of the respective reference sequences. Each genome sequence was submitted to GenBank for archiving. Virus diversity was analysed by percentage identity of the nucleotide sequence, and synonymous and non-synonymous substitution rates and Tajima's test of neutrality of the protein coding sequence. A neighbour joining phylogenetic tree was also inferred to compare the virus with closely related species.

3.2 Distribution of viruses infecting tree tomato

3.2.1 Development of RT-PCR diagnostics for detection of PVY, TMMoV, ETBTV and satRNA-E, and PSTVd

3.2.1.1 Primer design and annealing temperature optimisation

Primers were designed using NCBI's Primer BLAST program. Each genome that was assembled in Section 3.1.2.3 was used as a template and primers derived from them with several parameters: product size of between 300 to 1000 bp; and primer melting temperatures were to have an optimal of 60ºC with a minimum difference of 3ºC. The primer pair was checked for specificity against the GenBank virus database. Due to PVY being highly diverse, primers targeting a conserved region of the coat protein region of the species' genome were obtained from published data (Przewodowska *et al*., 2015). DreamTaq™ PCR master mix was used and annealing temperature optimisation was done by running a gradient on the cDNA pool sample that was kept aside as described in Section 3.1.2.2. A gradient PCR was run across three annealing temperatures ranging 3ºC, 5ºC, and 7ºC below the melting temperature of less

stable primer (primer with the lower melting temperature). Results were viewed on a 1% agarose gel which was prepared by measuring 1g of agarose and boiling in 100 ml of tris borate EDTA buffer (X0.5 TBE). The gel was allowed to cool to around 60°C, then 3µl of ethidium bromide was added and swirled gently to mix. The gel was then poured onto a casting tray and allowed to solidify. Four (4) µl of the RT-PCR product was run together with a ladder and the results visualized under the ultra-violet trans-illuminator and photos taken. The samples with the clearest band was used as the annealing temperature in further reactions.

3.2.2 Determination of virus incidence and distribution using RT-PCR and sequencing

3.2.2.1 Sample preparation and PCR analysis

Testing was done in two tiers: first on RNA pooled from samples per farm; and then a second tier where RNA from individual samples from a subsequent farm were tested for only the viruses which the farm pool tested positive for (Figure 3.1). In tier one, 5 µl of RNA extracted from each sample was pooled together with other samples from the farm where the samples were collected from. The concentration of the RNA pool was measured using a Nanodrop spectrophotometer prior to cDNA synthesis and diluted to 0.5 µg/µl. In each tier, 10 µl reactions were constituted according to manufacturer's protocol as follows: 5 µl of reaction mix, 0.2 µl of each forward and reverse primers (10 µM), 0.4 µl of the enzyme mix (SuperScript™ III RT and Platinum™ Taq mix), 2.2 µl of sterile water and 2 µl of the RNA template. The thermocycler was set as follows: reverse transcription was done at 50ºC for 30 minutes; initial denaturation at 94ºC for 2 minutes; cyclic denaturation at 94ºC for 20 seconds; annealing temperature (as determined in previous section) for 20 seconds; cyclic extension at 68ºC calculated at rate of 1kb/minute; final extension at 68ºC for 5 minutes; and storage at 12ºC. Annealing temperature for the PVY primers was 58ºC as is documented in the

publication (Przewodowska *et al*., 2015). Tier two reactions were set in a similar manner as tier one while testing the individual samples. Results were viewed on a 1% agarose gel.

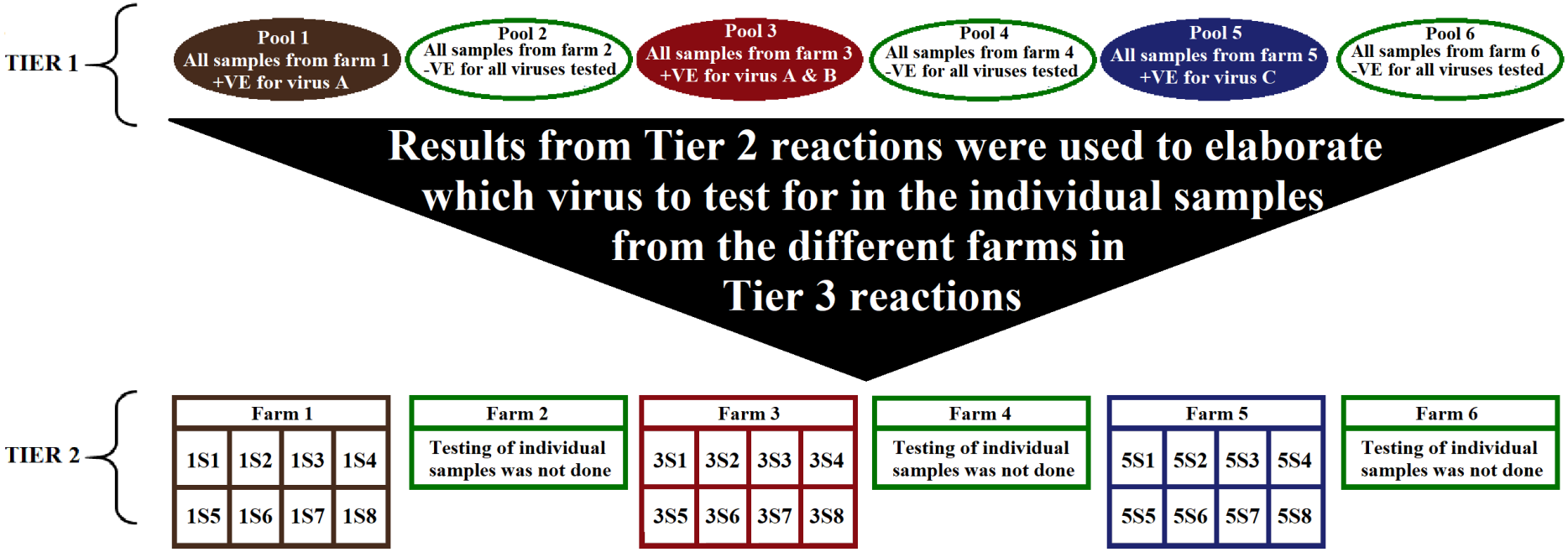


Figure 3.1 schematic representation of the two-tier system used in RT-PCR to test for viruses. The colour coding represents the different viruses detected at each stage.

3.2.2.2 Statistical analysis of virus incidence and distribution of disease

The number of plants positive for each virus tested in each farm were counted and the percentage incidence computed for each virus in each farm. Mean comparisons of the virus incidence in the different agro-ecological zones were done using student t-test at 95% confidence level. Virus distribution was mapped according to counties where the samples were collected.

3.2.2.3 Sequence analysis of RT-PCR products and NGS results validation

One positive sample for each virus from each farm was taken for Sanger sequencing. Sequencing was done on forward and reverse directions using the respective forward and reverse primers. Nucleotides with low quality scores were trimmed from the 5' and 3' ends. The forward and reverse sequences were aligned to each other using CLC genomics workbench and a consensus sequence derived. The consensus sequences were compared using BLAST to their respective NGS assembled genomes, and the whole GenBank database. Annotation of the protein coding sequences was done by comparing with their respective reference sequences and then submitted to GenBank. The PVY Sanger sequences were aligned to each other and the rates of the synonymous and non-synonymous substitution determined using Tajima's test of neutrality, and a phylogenetic tree inferred.

CHAPTER 4 RESULTS

4.1 Identification and characterization of viruses infecting tree tomato in Kenya

4.1.1 Surveys for viruses infecting tree tomato in Kenyan farms

4.1.1.1 Social economics of tree tomato farmers in surveyed orchards

A survey on tree tomato virus diseases was conducted in select tree tomato growing areas in Kenya in: November to December 2018 2019 in Machakos, Nairobi, Embu, Tharaka Nithi, and Meru; and in August 2019 in Elgeyo Marakwet, Nandi, Baringo and Nakuru.

During the survey, it was noted that tree tomato farming was done by both men and women at 73% and 27%, respectively (Table 4.1). The orchards surveyed were categorized as either small scale (9 > 100 trees) or large-scale (100 < 7000 trees). The small scale farms comprised 69% while the large comprised 31%. All the female farmers plus 58% of their male counterparts managed small scale orchards while the 42% of the male farmers managed large scale orchards. All the small scale famers did it both for subsistence and for sale at parochial markets, while the large scale farms had access to the city markets in Nairobi and Nakuru, and the international markets. Prices ranged between Kenya shillings 50 and 90 per kilogram for both small- and large-scale farmers. Small scale famers raised seedlings from seeds obtained from fruits they bought from the market and while others obtained them from neighbors. The large-scale famers obtained their seedlings from seedling vendors. The large-scale farmers were unaware of the certification status of the seedlings they bought from the vendors.

The main constraints identified by the famers included: bad weather such as drought and hail; diseases which included powdery mildew, die back, root rot; pests including aphids, white flies, and cut worms; and weeds which included black jack, amaranthus and datura were present in some of them (Table 4.1). There was small proportion of small scale to large scale farms using pesticides, 27% and 62.5% respectively. The types of pesticides applied included: insecticides

(thiamethoxam, cypermethrin, carbosulfan, abamectin, lambda-cyhalothrin, acetamiprid); and fungicides (mancozeb, cabendazim, azoxystrobin, difenoconazole, metalaxyl). There was small proportion of small scale to large scale farms using soil amendments, 39% and 62.5% respectively. The soil amendments applied CAN, urea, 17:17:17, agromaster, 10-10-10, lime, and lamilawina, and manure.

All of the farmers practiced intercropping with potatoes, tomatoes, capsicum, tobacco, peppers, kale, avocado, arrow roots, cassava, apples, coffee, maize, macadamia, sweet potato, banana, beans, oranges and tea. These weeds and other crops with which tree tomato was intercropped could to serve as potential virus reservoirs for most of the viruses known to infect tree tomato. None of the farmers had access to certified virus free seeds or seedlings.

Table 4.1 Summary of the demographics of tree tomato farms

Male farmers	**Female farmers**	**Small-scale farms**	**Large-scale farms**
73% male Commercial farming	27% female Subsistence farming	$9 \geq 100$ trees Seedlings from seedling vendors Subsistence farming and selling in parochial markets	$101 < 7000$ trees Seedlings from market bought fruits Selling in city markets in Nairobi and Nakuru, and international markets
Constraints	**Pesticide**	**Soil amendment**	**Alternative crops**
Weeds (amaranthus, black jack, and datura), disease (powdery mildew, die back, root rot), drought, hail, wind, insect pests (aphids, whiteflies, cutworms)	Mancozeb, metalaxyl, cabendazim, abamectin, azoxystrobin, difenoconazole, thiamethoxam, cypermethrin, carbosulfan, cyhalothrin, acetamiprid	Manure, CAN, urea, 17:17:17, lime, agromaster, 10-10-10, lamilawina	Potatoes, tomatoes, kale, capsicum, tobacco, maize, peppers, avocado, arrow roots, beans, apples, sweet potato, tea, coffee, banana, macadamia, oranges

4.1.1.2 Sample collection and assessment of virus associated disease symptom

A total of 358 trees were recorded and samples collected from three agro-ecological zones; upper highlands, lower high lands and midlands (Table 4.2). Leaves from one tree constituted one sample.

Table 4.2 Location of the farms from where the survey was conducted

County	Sub-County	Ward	Farm	GPS		Altitude	Agro-ecological zones	Number of samples examined	Number of samples collected	Samples code
				Latitude	Longitude					
Machakos	Kathiani	Upper Kaewa	1	-1.4725	37.3135	1850	Lower Highlands	10	5	T1-T5
			2	-1.4711	37.3135	1872	Lower Highlands	20	6	T6-T11
			3	-1.4721	37.3109	1895	Lower Highlands	30	14	T12-T25
Embu	Manyatta	Mbeti North	4	-0.5541	37.4958	1278	Midlands	20	7	T27-T33
			5	-0.5482	37.4863	1307	Midlands	30	14	T34-T47
	Runyenjes	Kagaari South	6	-0.4719	37.5664	1369	Midlands	30	13	T48-T60
		Kagaari North	7	-0.3915	37.5447	1657	Midlands	20	8	T61-T68
			8	-0.3898	37.5458	1412	Midlands	20	7	T69-T75
Tharaka-Nithi	Maara	Chogoria	9	-0.2034	37.6184	1653	Midlands	20	6	T76-T81
			10	-0.2409	37.5996	1675	Midlands	50	18	T82-T99
			11	-0.2450	37.6169	1629	Midlands	40	11	T100-T110
		Mwimbi	12	-0.2124	37.6898	1262	Midlands	30	12	T111-T121
Meru	Imenti South	Abogeta West	13	-0.0972	37.5733	2048	Lower Highlands	30	11	T122-T132
			14	-0.0982	37.58	2046	Lower Highlands	30	10	T133-T142
Nairobi	Westlands	KALRO	15	-1.2586	36.7745	1788	Midlands	20	10	T143-T152
Elgeyo Marakwet	Keiyo North	Kamaring	16	0.5448	35.475	2507	Upper Highlands	20	6	T153-T158
		Kaptarakwa	17	0.5210	35.4897	2482	Upper Highlands	30	14	T159-T172
			18	0.4819	35.5280	2572	Upper Highlands	80	25	T173-T197
Nandi	Emgwen	Kapsabet	19	0.1974	35.1218	2018	Lower Highlands	80	28	T198-T225
			20	0.2047	35.1379	1955	Lower Highlands	40	17	T226-T242
	Chesumei	Kosirai	21	0.3277	35.1834	2102	Lower Highlands	80	39	T243-T281
			22	0.294	35.1746	2105	Lower Highlands	40	14	T282-T295
Baringo	Koibatek	Sirwa	23	0.1906	35.7826	2124	Lower Highlands	40	16	T296-T311
		Eldama Ravine	24	0.0053	35.5732	2610	Upper Highlands	80	26	T312-T337
			25	0.0516	35.5647	2781	Upper Highlands	100	19	T338-T356
Nakuru	Naivasha		26	-0.7019	36.4301	1906	Lower Highlands	10	2	T357-T358

All farms in Machakos showed symptoms commonly associated with virus infections such as mottling, vein clearing and leaf malformation (Figure 4.1A-B). The samples collected at the KALRO, Biotechnology Centre farm in Nairobi showed symptoms of leaf malformation and curling and vein banding (Figure 4.1C).

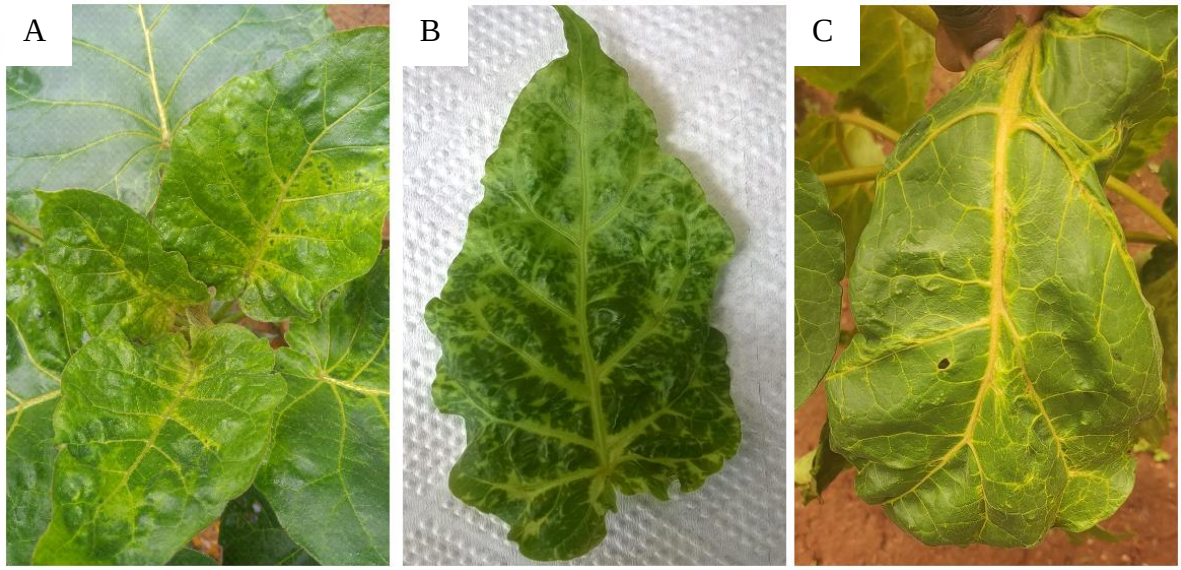


Figure 4.1 Leaf samples showing mottling and vein clearing (**A** and **B**) from Machakos, and vein banding and leaf curling (**C**) from Nairobi counties.

Tree tomato trees in Embu County also showed symptoms of vein banding, yellowing and necrosis, depressions on the lamina of leaves, leaf curling and mottling on leaves and water-soaked blemishes on the fruits, as well as aphid infestation in some of the orchards (Figure 4.2). Orchards in Tharaka Nithi and Meru counties showed several symptoms including leaf malformation, vein banding and leaf rolling, yellowing, brown lesions on leaves and stems, and die back (Figure 4.3). Heavy infestation of aphids in some of the farms in these counties was also noted (Figure 4.3E). Orchards in Elgeyo Marakwet, Nandi and Baringo showed leaf malformation and curling (Figure 4.4 and 4.5).

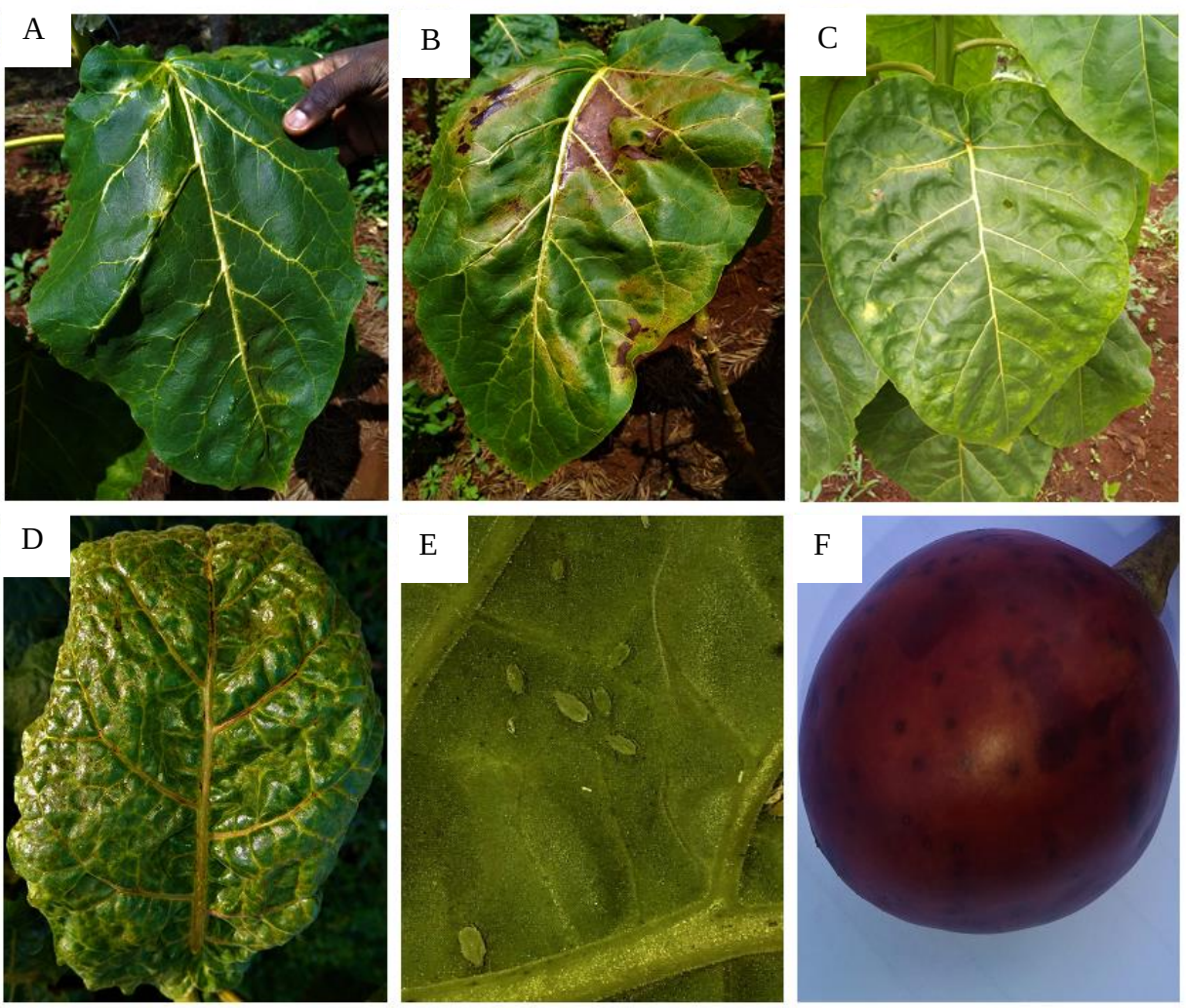


Figure 4.2 Tree tomato displaying virus symptoms in Embu county **A** – vein banding; **B** – vein banding progressing into necrosis; **C -** lamina depressions; **D** – leaf curling and puckering; **E** – aphid infestation on the underside of the leaf; **F** – water soaked blemishes on fruit surface

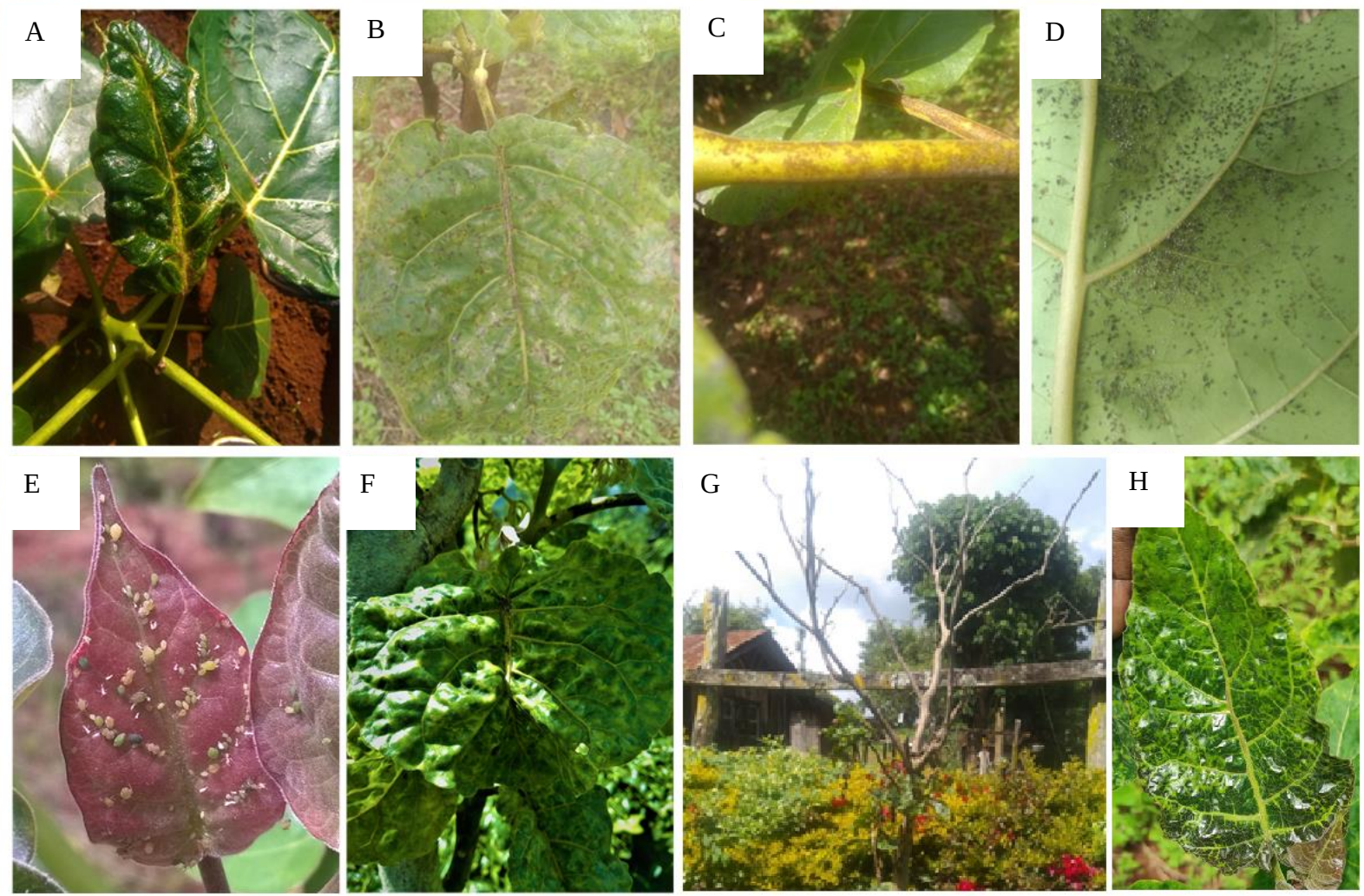


Figure 4.3 Tree tomato plants from Tharaka Nithi (**A** – **E**) and Meru (**F** – **H**) showing diverse disease symptoms including: **A** – vein banding and leaf curling; **B** and **C** showing - brown lesions on leaves and green branches; **D** and **E** – heavy aphid infestation; **F** – yellowing and leaf malformation; **G** – die back; **H** – vein clearing with dark green islands

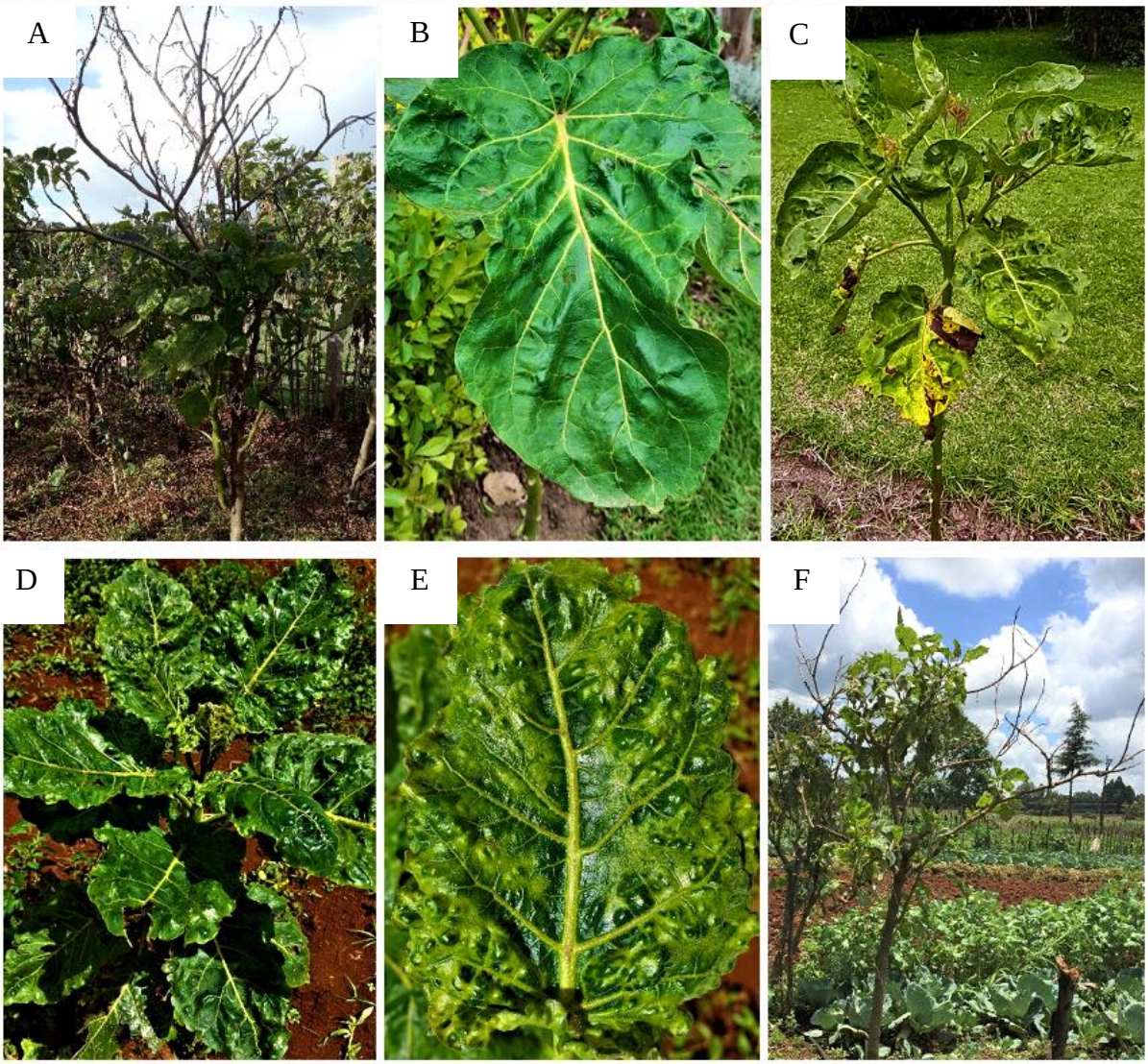


Figure 4.4 Tree tomato plants in Elgeyo Marakwet (**A** – **C**) and Nandi counties with disease symptoms: **A** – dieback; **B** – leaf malformation; **C** – leaf curling, yellowing and necrosis, **D** – leaf malformation; **E** – crinkling with dark green islands; **F** – and die back.

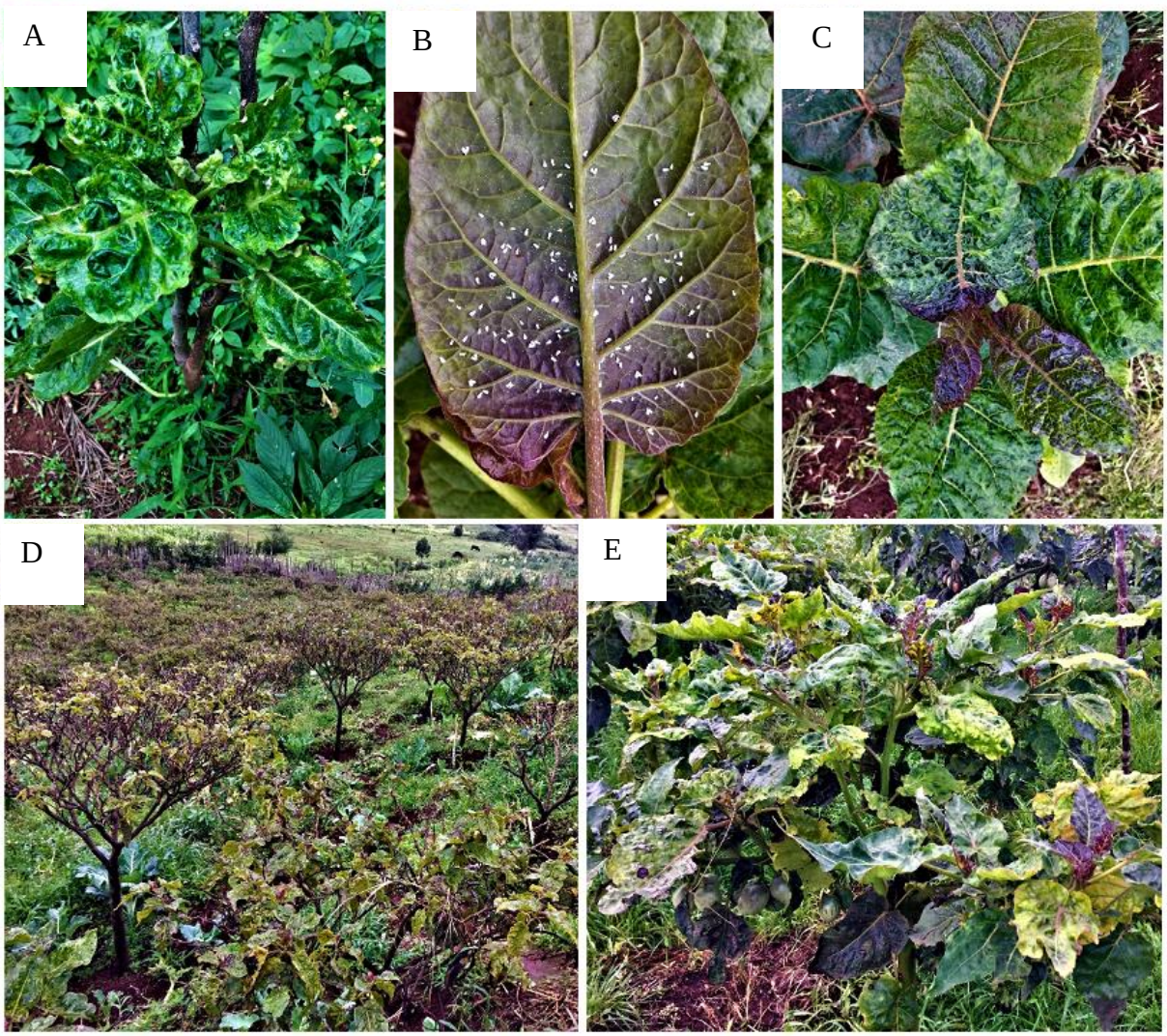


Figure 4.5 In Baringo County, symptoms in this farms included: **A** – leaf malformation, yellowing with dark green islands; **B** – whitefly infestation on the underside of a leaf; **C** – yellowing and leaf malformation; **D** and **E** – field ravaged by yellowing and dieback symptoms

Disease prevalence was 100% for all regions (Table 4.3). Most of the farmers did not practice any disease or pest management while those who did, still had diseased plants. Average disease incidence of the various counties surveyed ranged between 60-80% while severity ranged between 2.1 and 3.5. Baringo County had the highest incidence while Meru had the highest severity, followed by Nairobi, Tharaka Nithi and Embu (Table 4.3).

Table 4.3 Prevalence, incidence and severity of virus symptom in tree tomato plants in the major production areas in Kenya during 2018-2019 growing season, and the honest significant difference (HSD)

County	**Number of trees examined**	**Prevalence**	**Incidence**	**Severity**
Embu (ML)	120	100%	70%	2.6
Tharaka-Nithi (ML)	140	100%	65%	3
Nairobi (ML)	20	100%	60%	3.2
Machakos (LH)	60	100%	70%	2.3
Meru (LH)	60	100%	70%	3.5
Nandi (LH)	240	100%	75%	2.4
Nakuru (LH)	10	100%	80%	2.1
Baringo (F23) (LH)	180	100%	80%	3.15
Elgeyo Marakwet (UH)	130	100%	70%	2.2
Baringo (F24-25) (UH)	40	100%	80%	2.4
P<0.01			0.35	1.406e-32
Significance			Ns	*
Tukey-Kramer of Severity differences	**ML/LH**	**LH/UH**	**UP/ML**	
Q calculated	3.46	14.61	16.43	
HSD (α=0.01)				4.13

* Significance level at p < 0 .01; ns: no significant difference, ML – midlands; LH – lower highlands; UH – upper highlands.

A one-way ANOVA showed that there was a significant difference ($p<0.01$) in the severity between the ML, LH and UH and none in the incidence (Table 4.2). A Tukey-Kramer post hoc test ($\alpha<0.01$) indicated that the difference observed was between the UH and the other two, ML and the LH. There was no difference in severity observed between the midland and the lower highland.

4.1.2 Identification and characterization of viruses infecting tree tomato

4.1.2.1 Sequencing analysis

Sequencing from the ion torrent resulted in 9 million reads and with 8 million reads remaining after trimming and quality control. Most of the sequences lengths were distributed between 79 – 200 bp (Figure 4.6).

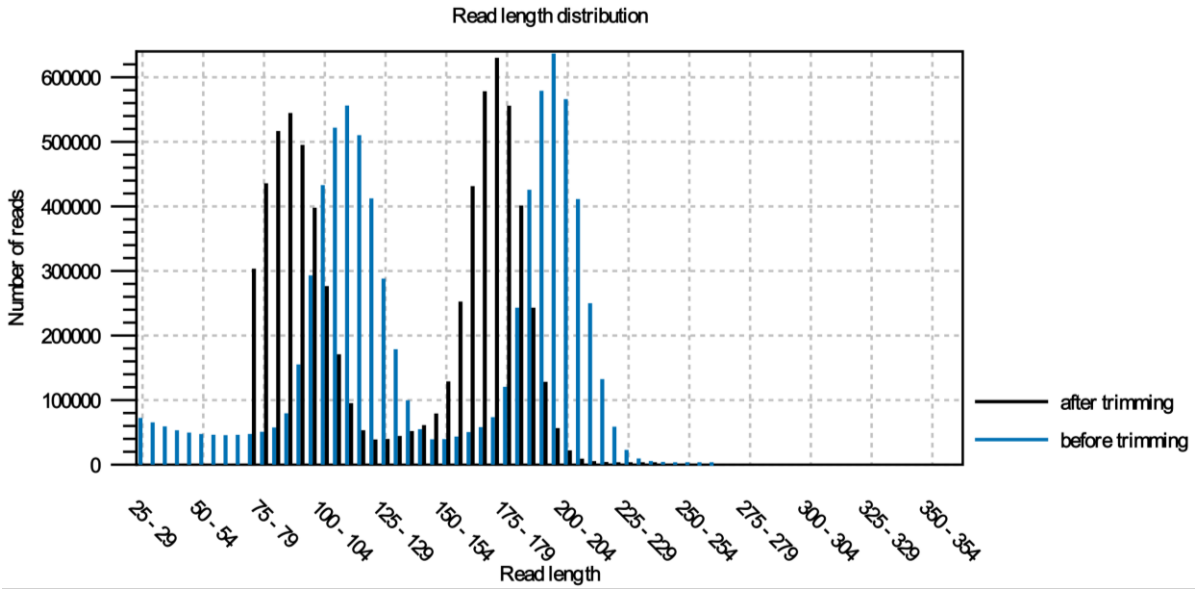


Figure 4.6 Summary of read length distribution before and after trimming

Initial analysis determined three viruses, a satellite RNA, and a viroid to be present: potato virus Y (PVY); tomato mild mottle virus (TMMoV); Ethiopian tobacco bushy top virus (ETBTV) and its associated RNA satellite; and potato spindle tuber viroid. With the exception of PVY, the reads aligning to each were used to perform a reference guided genome assembly. Further analysis revealed reads aligning to other viruses including; pepper enomavirus, tobacco mottle virus, tomato chlorosis virus, Kenyan potato cytohabdovirus and tamarillo fruit ring virus. Research is ongoing to confirm the presence of these viruses using other RT-PCR.

4.1.2.2 Potato virus Y

A total of 1572 reads aligned with genomes of the members of the PVY complex. The reads mapped to several PVY strains and recombinants. A PVY genome was thus not established as it would have led to building a non-existing chimeric strain. A more stringent mapping against the major five parent strains, C, Eu-N, NA-N, O5 and O, did not shed light or assist in strain identification. The reads showed high percentage alignment to: strain O from the 5’ to 3’ end; strain C from 1200 bp to 3’ end; and strains Eu-N and NA-A shared most reads which aligned at between 5500 – 6000 bp, and 8700 bp and 9300 bp.

4.1.2.3 Tomato mild mottle virus

BLAST results showed 2362 reads aligned to TMMoV. A reference guided genome assembly of TMMoV was done using Ethiopian isolate NC038920 enabling assembly of a complete coding sequence (CDS) of 9243 base pair (bp) in length (Figure 4.7). This genome was submitted to NCBI GenBank under accession number MW537585. A query of the whole genome against the GenBank database revealed a 97.09% identity similarity to the exemplar strain (NC038920) and a 98.13% to a nucleotide segment of the coat protein gene (HQ711860) collected from Kenya.

The genome of MW537585 is organized in a similar manner to that of NC038920: two non-translated regions (NTR) at the 73 bp and 135 bp, at the 5' and 3' ends respectively; two open reading frames (ORF): a 9036 bp region coding for a 3011 amino acid (aa) sequence of the polyprotein gene in the middle flanked by the two NTR regions; and the other being 252 bp overlapping the P3 peptide region, and coding for 84 aa of the PIPO gene (Figure 4.8). Alignment of the different genes of MW537585 to the known strains, TMMoV-E (Ethiopian, NC038920) and TMMoV-I (Israeli, HQ840786), showed little diversity with percentage identities ranging between 94–99% against the former, while and 79–83% against the latter with exception of the PIPO gene which showed the highest conservation at 89% (Table 4.4A). Non-synonymous changes were constrained in the polyprotein gene (Table 4.4B).

Table 4.4 Part **A** percentage identity similarity of known genes and Part **B** the synonymous and non- synonymous ratio (**dN/dS**) of the Kenyan isolate of tomato mild mottle virus (TMMoV, accession number MW537585) to the known strains, Ethiopian (NC038920), and Israeli (HQ840786).

A	Gene	Polyprotein	P1	Hc_Pro	P3	6K1	CI
	NC038920	97	97	97	97	97	97
	HQ840786	81	79	80	82	79	82
MW537585	Gene	6K2	Vpg	Nia	Nib	CP	PIPO
	NC038920	94	96	98	97	97	98
	HQ840786	83	83	81	80	81	89

B	Polyprotein	Synonymous differences (dS)	Non- synonymous differences (dN)	dN/dS
MW537585	NC038920	0.1084	0.0083	0.076568
	HQ840786	2.1792	0.0456	0.020925

The predicted cleavage sites of the polyproteins into the mature peptides showed a high amount of conservation with only single amino acid changes across the different genes of the members of the species (Table 4.5). The polyprotein sequence indicated a similar organisational pattern to the known TMMoV strains (Figure 4.8).

Table 4.5 Putative cleavage sites identified in the polyproteins of the available TMMoV isolates from the different regions

Peptide junction	HQ840786	NC038920	MW537585
	'/' cleavage site		
P1/HC-Pro	DIEFY/AKSDI	DVEFY/AKSDI	DVEFY/AKSDI
HC-Pro/P3	YSIGG/GLVTD	YSIGG/GLVTD	YSIGG/GLATD
P3/6K1	VLETH/SKEKE	VLETH/SKEKE	VLETH/SKEKE
6K1/CI	RVYVQ/HGSWA	RVYIQ/HGSWA	RVYIQ/HGSWA
CI/6 K2	YLEAH/SKQEF	YLEAH/SKQEF	YLEAH/SKQEF
6K2/Vpg	ELELH/GKSKR	ELELH/GKSKR	ELELH/GKSKR
Vpg/NIa	KLEEH/SGVNE	KLEEH/SGVNE	KLEEH/SGVNE
NIa/NIb	PIEVH/SAPSN	PIEVH/SAPGN	PIEVH/SAPGS
NIb/CP	SLEEH/SGSIA	SFEEH/SSSIA	SFEEH/SSSIA

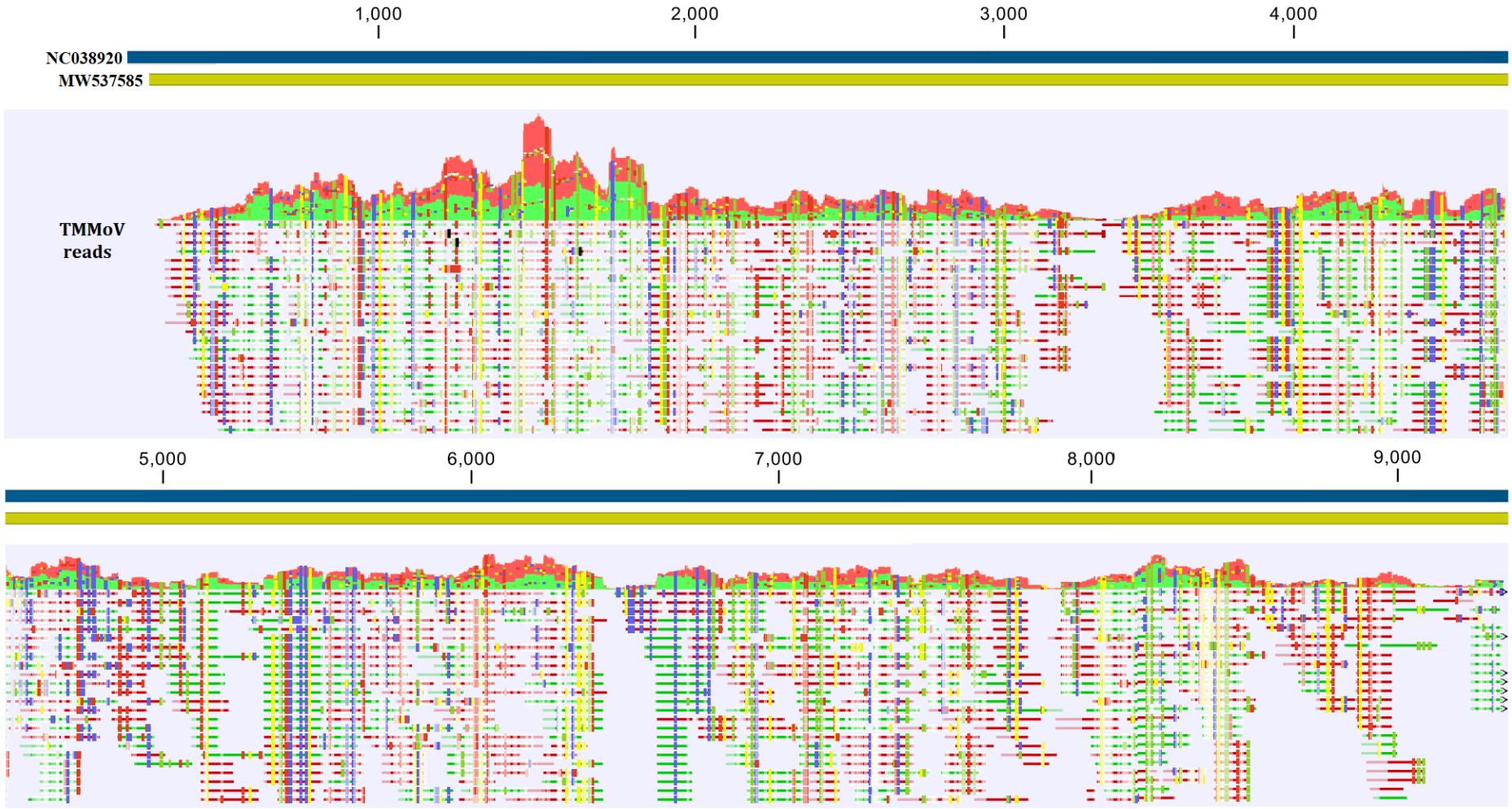


Figure 4.7 Mapping of reads to tomato mild mottle virus (TMMoV) reference sequence NC038920 (blue bar) producing the consensus sequence MW537585 (green bar). The different coloured bars underneath represent the various reads aligned at the specific positions

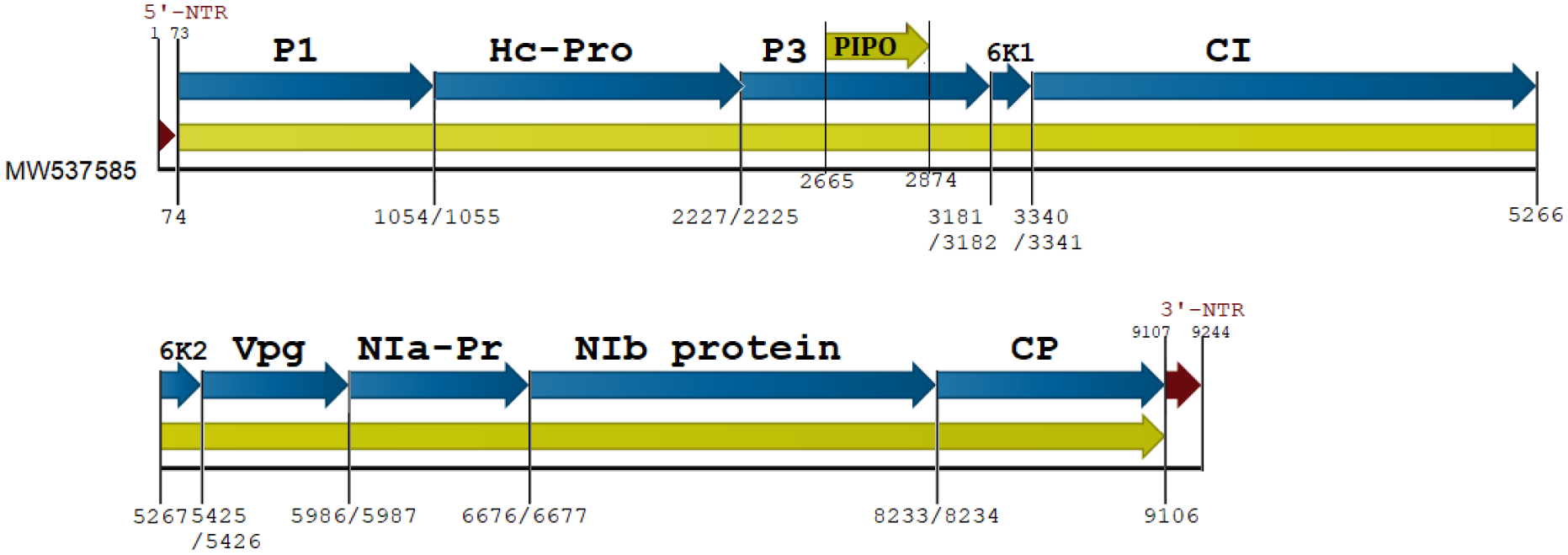


Figure 4.8 Genome organization of the Kenyan isolates of tomato mild mottle virus (TMMoV). The image is as follows: black line underneath – whole sequence; the yellow arrow – polyprotein gene and the PIPO protein; the numbers underneath – genome annotations; and the blue arrow – peptides with their names written on top; and the red and brown arrows – 5' and 3'non-translated (NTR) region.

A phylogenetic tree showed close relation to the two TMMoV genomes, one from Ethiopia and another from Israel (Figure 4.9). The Kenyan isolate was most similar to the Ethiopian strain, NC038920-E. The tree showed three distinct sub-groups based on the presence of certain genes (Dombrovsky *et al*., 2014): sub-group 1 are characterised by the presence of a HC-pro gene; sub-group II viruses are viruses containing a tandem repeat of the P1 protein sequence; and sub-group III are viruses which encode the HAM1-like protein.

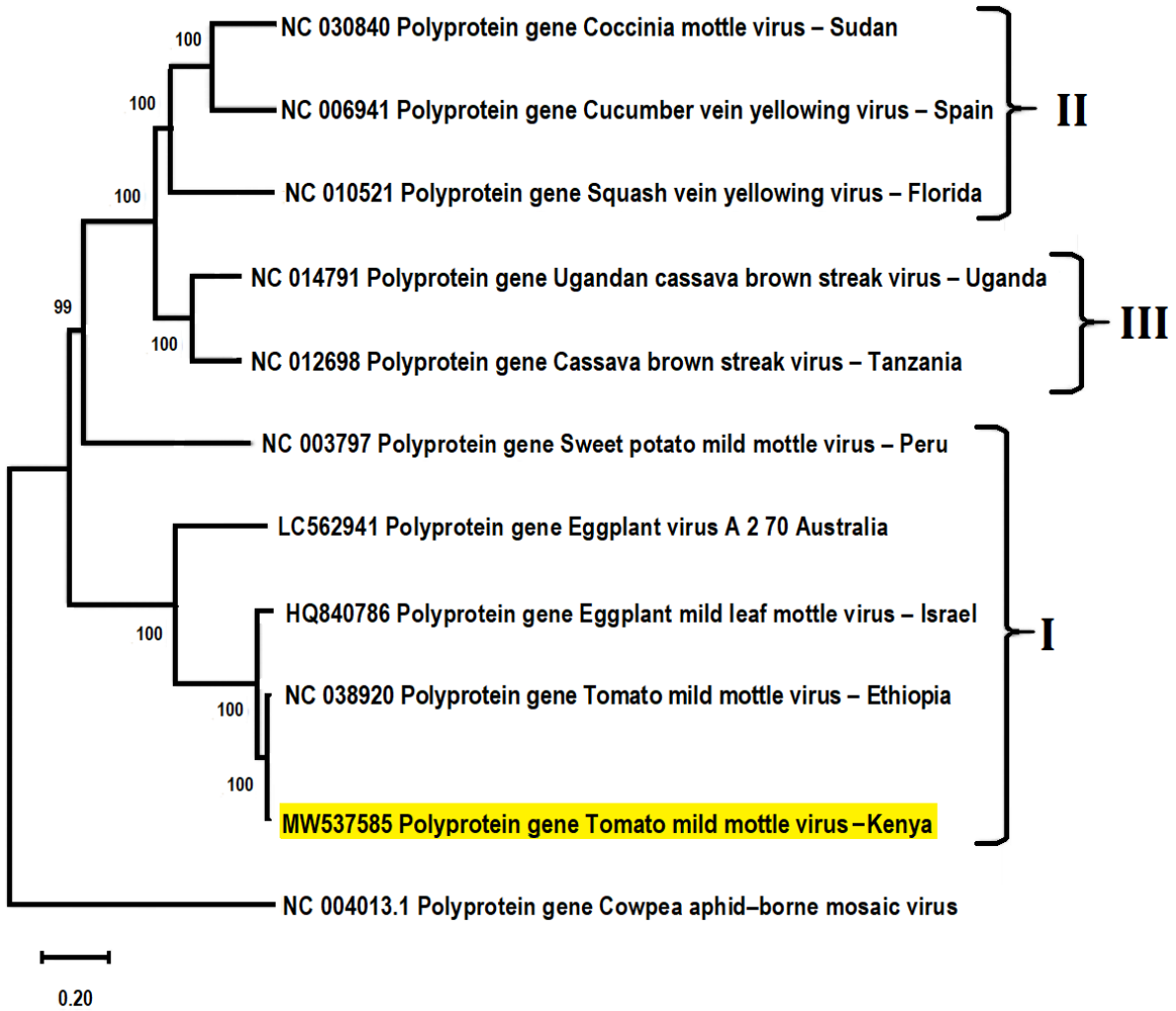


Figure 4.9 A neighbour-joining tree constructed with MEGA 11 using 1000 bootstrap replicates for polyproteins of various members of the genus Ipomovirus. Cowpea aphid-borne mosaic virus (NC004013) was used as the outgroup. The Kenyan isolate MW537685 is marked in yellow.

The Kenyan isolates in this study indicate that there is a low divergence of the genetic code of the virus especially when compared to the Ethiopian one at 96% but quite distinct from the Israeli one at 81%. On the gene level, the polyprotein showed very little diversity in the nucleotide sequence coding.

4.1.2.4 Ethiopian tobacco bushy top virus and associated satellite

BLAST results showed that 236 reads aligned to Ethiopian tobacco bushy top virus (ETBTV; GenBank Accession No. NC024808), 489 aligned to ETBTV-associated satellite RNA (satRNA; NC024807). Reference-guided genome assembly was done using the exemplar strains NC024808 and NC024807 for ETBTV and satRNA-E, respectively (Figure 4.10 – 11). This resulted in a partial genome for ETBTV (4199 bp; MW883068), and a full genome of the satRNA-E (522bp; MW713579). Annotation using the exemplar strain showed a similar genome organisation to that of the other isolates, and consisted of four ORF's coding for proteins as follows: replication-associated protein; RNA-dependent RNA polymerase (RdRp); long-distance movement protein; cell-to-cell movement protein. There was a long 3' NTR, over 600 bp (Figure 4.12)

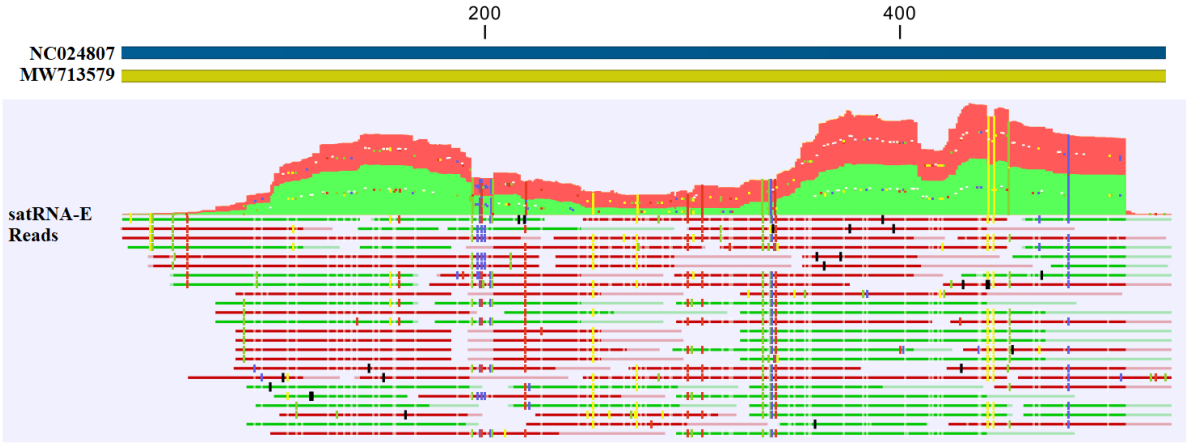


Figure 4.10 Mapping of reads to satRNA-E reference sequence NC024807 (blue bar) producing the consensus sequence MW713579 (green bar). The different coloured bars underneath represent the various reads that aligned at the specific positions.

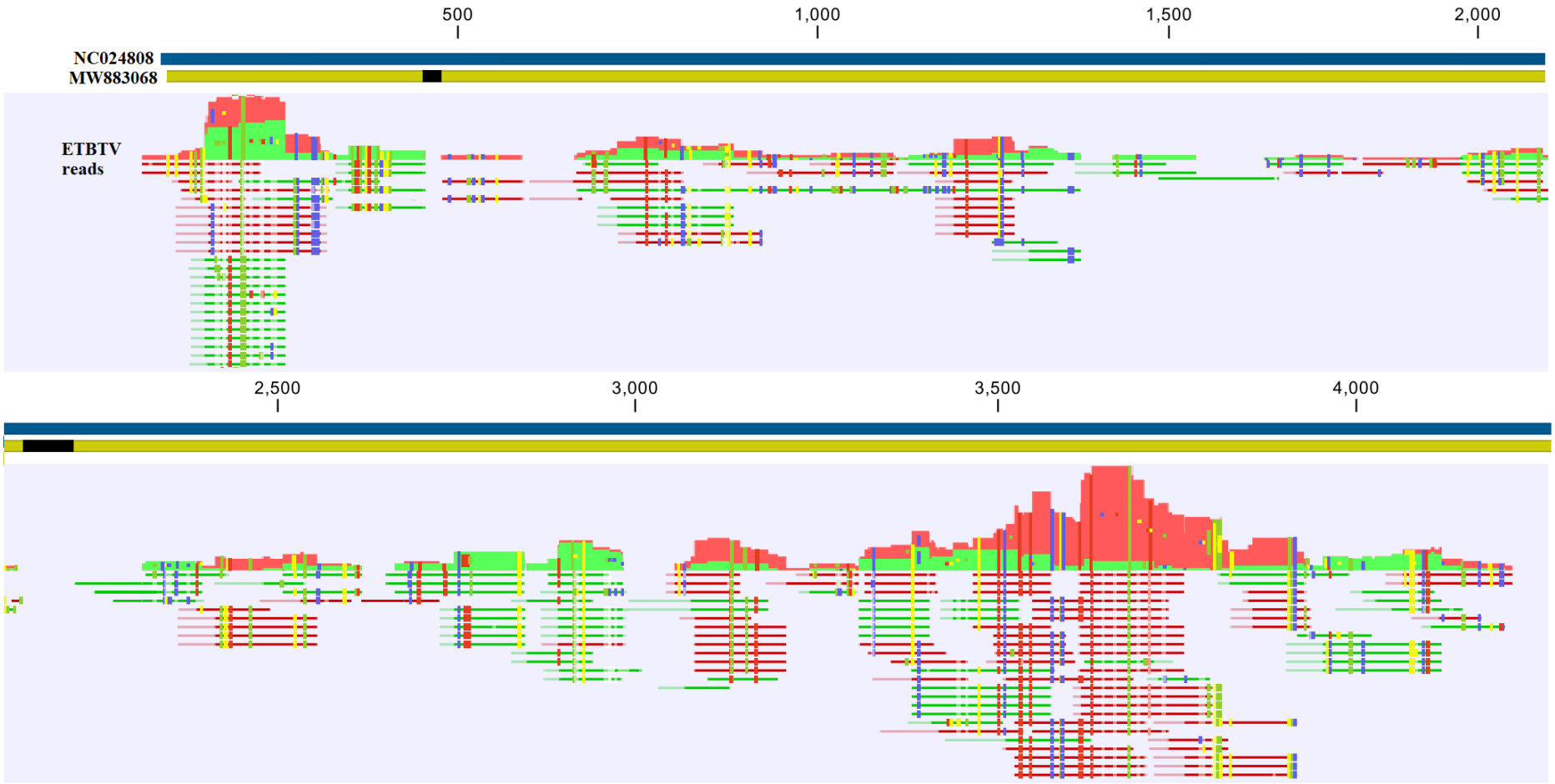


Figure 4.11 Mapping of reads to Ethiopian tobacco bushy top virus (ETBTV) reference sequence NC024808 (blue bar) producing the consensus sequence MW883068 (green bar). There were two gaps (black bar interrupting the green bar), at positions 416 – 438 and 2123 – 2299. The different coloured bars underneath represent the various reads aligned at the specific positions.

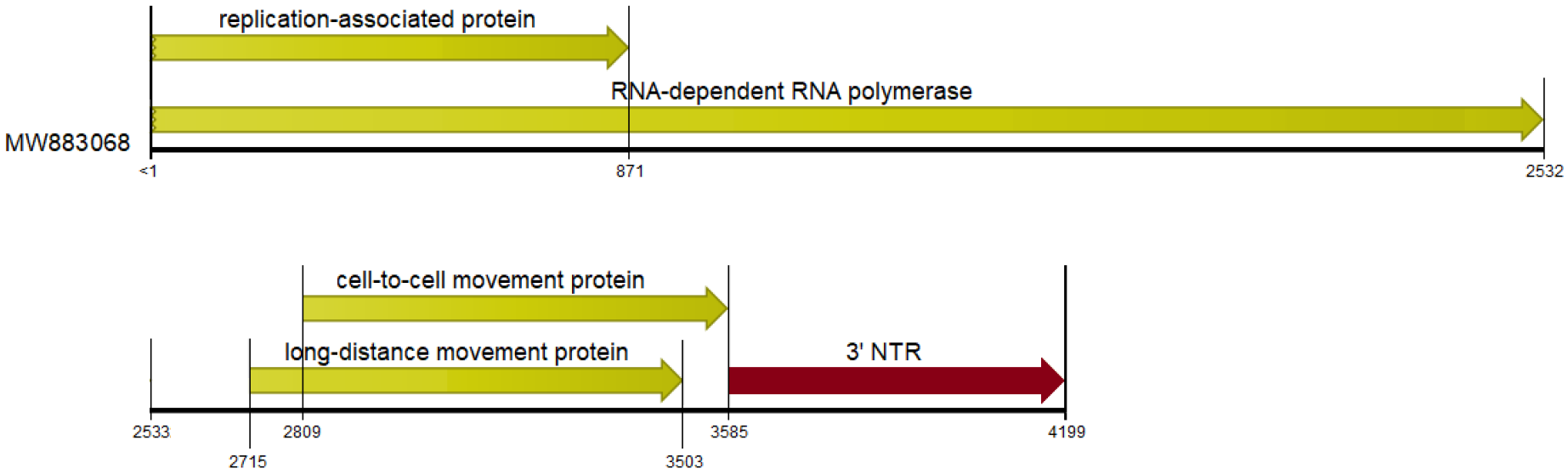


Figure 4.12 Schematic representation of the genome organization of the Kenyan isolates of Ethiopian tobacco bushy top virus (ETBTV). The genome codes for four proteins (designated by yellow arrow with the name of the protein on top). The red arrow represents the 3' non translated (NTR) region.

Genome nucleotide alignments and phylogenetic analysis showed that MW883068, was most closely related to NC024808 (Ethiopian isolate) at 95% sequence identity, while the satRNA MW713579 had 98% identify to MT225093 and MT225092 (Zambian isolates). Gene sequences comparison with the other two ETBTV isolates from Zambia and Malawi (MT225089 and LC494673, respectively) showed high conservation, of 91% to 96%, on the region coding of two overlapping genes, long-distance movement protein and cell-to-cell movement protein (Table 4.6).

Table 4.6 Percentage comparison of the different genes coded in the Ethiopian tobacco bushy top virus (ETBTV) genome.

Virus Isolate	Replication-associated protein	RNA-dependent RNA polymerase	Long-distance movement protein	Cell-to-cell movement protein
NC024808	90.13%	90.91%	96.07%	95.62%
LC494673	83.20%	87.46%	91.23%	90.09%
MT225089	83.45%	87.32%	91.74%	91.49%

A phylogenetic tree (Figure 4.13) that was drawn using MEGA 11 showed that the Kenya isolate of ETBTV, KenVTT, was most closely related to the Ethiopian strain (NC024808). The associated RNA satellite had a percentage identity compared to other isolates from Zambia, 98% and 96% (MT225092, MT225093, MT225094), and least similarity, 95% with the Ethiopian Isolate (KJ918747).

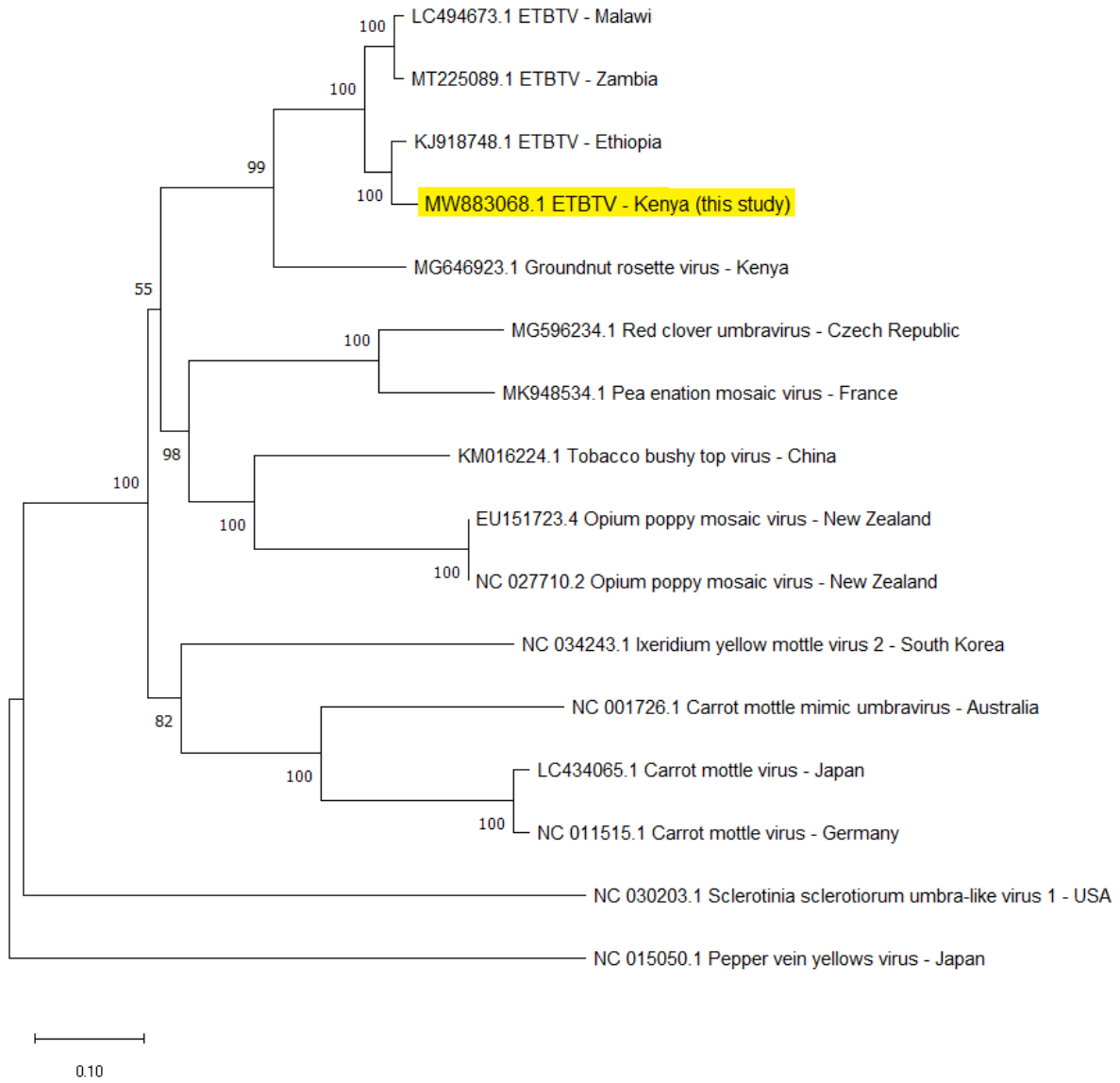


Figure 4.13 A neighbour-joining tree constructed with MEGA 11 using 1000 bootstrap replicates for polyproteins of various members of the genus *Umbravirus*. Pepper vein yellows virus was used as the outgroup. The Kenyan isolate MW883068 is marked in yellow.

4.1.2.5 Potato spindle tuber viroid

BLAST results revealed 26 reads aligning to potato spindle tuber viroid (PSTVd). A reference guided genome assembly was performed using the exemplar strain GenBank Accession No. NC002030 (Figure 4.14). This resulted in a 359 bp consensus genome sequence, which was added to GenBank MZ054164). BLAST results showed a 97.78% nucleotide alignment to a PSTVd isolate from the USA (JX280944). Phylogenetic analysis with select PSTVd isolates from other countries showed clustering with the USA isolate (Figure 4.15).

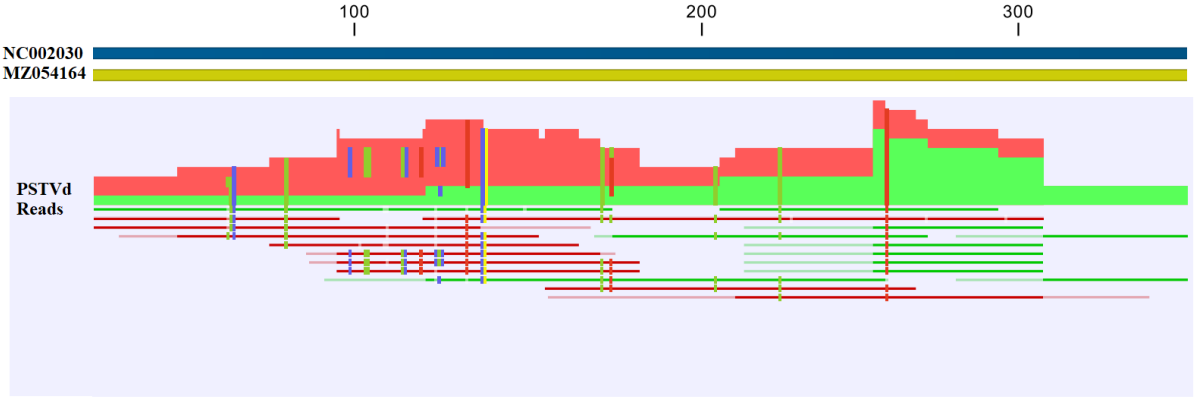


Figure 4.14 Mapping of reads to potato spindle tuber viroid (PSTVd) reference sequence NC024807 (blue bar) producing the consensus sequence MW713579 (green bar). The different coloured bars underneath represent the various reads that aligned at the specific positions

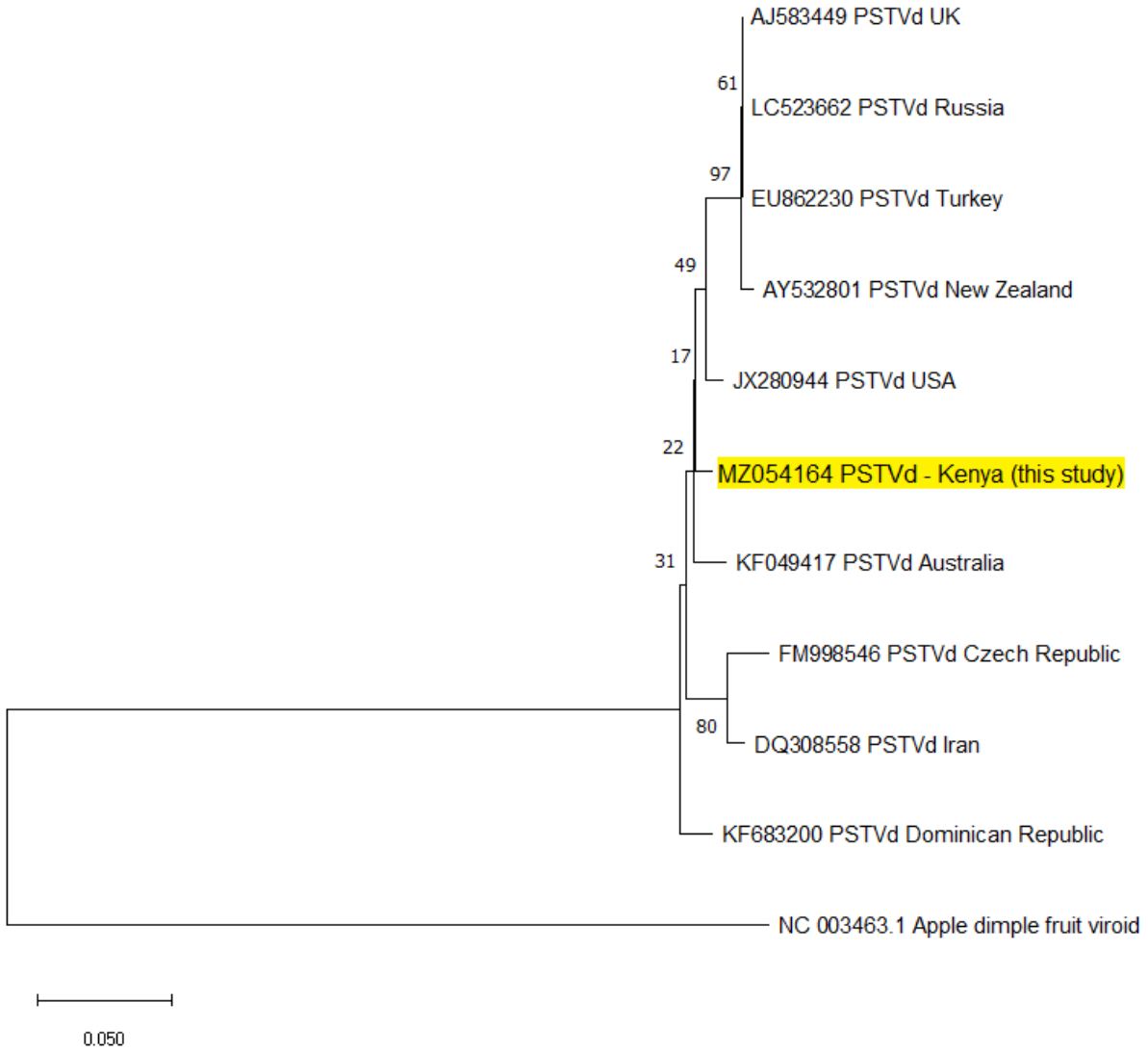


Figure 4.15 A neighbour-joining tree constructed with MEGA 11 using 1000 bootstrap replicates for isolates of PSTVd from various regions. Apple dimple fruit viroid was used as the outgroup. The Kenyan isolate MZ054164 is marked in yellow.

4.1.2.6 Other viruses detected but not characterized

Further analysis of the BLAST alignment data revealed other viruses whose reads were found and fragments of the genomes assembled. Some of these viruses like pepper enamovirus (PeEV), tobacco mottle virus, and tomato chlorosis virus were discovered late into the project and research is still ongoing to characterise and authenticate their presence in tree tomato using RT-PCR. The other two viruses, Kenyan potato cytohabdovirus and tamarillo fruit ring, have only been recently discovered in Kenya and Rwanda respectively, and BLAST analysis showed their potential presence after updating the virus database (2021) from the previous one (2019). Attempts at assembling of the genomes have resulted only in partial genomes and fragments (Table 4.7).

Table 4.7 Other viruses which were detected, number of reads which aligned, and the percentage of the genome covered by the reads

Virus	Number of reads	Percentage of genome assembled
Pepper enamovirus	487	80%
Tobacco mottle virus	710	80%
Tomato chlorosis virus	6	2%
Kenyan potato cytohabdovirus	7	5%
Tamarillo fruit ring virus	29	20%

4.2 Detection and distribution of viruses infecting tree tomato using RT-PCR

4.2.1 Primers design and annealing temperature optimisation

Primer BLAST resulted in 4 primer pairs for detection of TMMOV, ETBTV, SatRNA-E and PSTVd (Table 4.8). In determining the annealing temperature of each primer pair, the median temperatures of the gradient PCR, s2, E2, T2, and P2 showed clearer bands and thus were used for all subsequent reactions (Figure 4.16). The RNA pool kept aside in section 3.1.2.2, was used as the source for positive control for all viruses tested.

Table 4.8 List of primers used in this study

Virus	Primer name	Sequence (5'->3')	T_a (°C)	Size (bp)	Target Region	Reference
TMMOV	Tom_F Tom_R	TGTGGGCCTACGACTTTAAC GGCTCCCATAGAAGCGTGTT	54	668	NIa-NIb	This study
ETBTV	ET_F ET_R	CGCACATGATTGCCATGGAG TTGTACAGTTTGCCCAACGC	55	789	RdRp	
SatRNA-E	Sat_F Sat_R	GTGAAAACGTCACCCCAGC AGCACCGCCCTCACGG	55	405	Partial genome	
PSTVd	Pd_F Pd_R	CGGAACTAAACTCGTGGTTCCTG AGGAACCAACTGCGGTTCCAA	56	~359	Whole genome	
PVY	F3 B3	CGTTGAAACCAATCGTTGAGAA GACATCCTCGGTGGTGTG	58	332	CP	Przewodowska *et al.* (2015)

T_a – annealing temperature; NIa – Nuclear Inclusion protein a; NIb – Nuclear Inclusion protein b; RdRp – RNA dependent RNA polymerase; CP – Coat protein

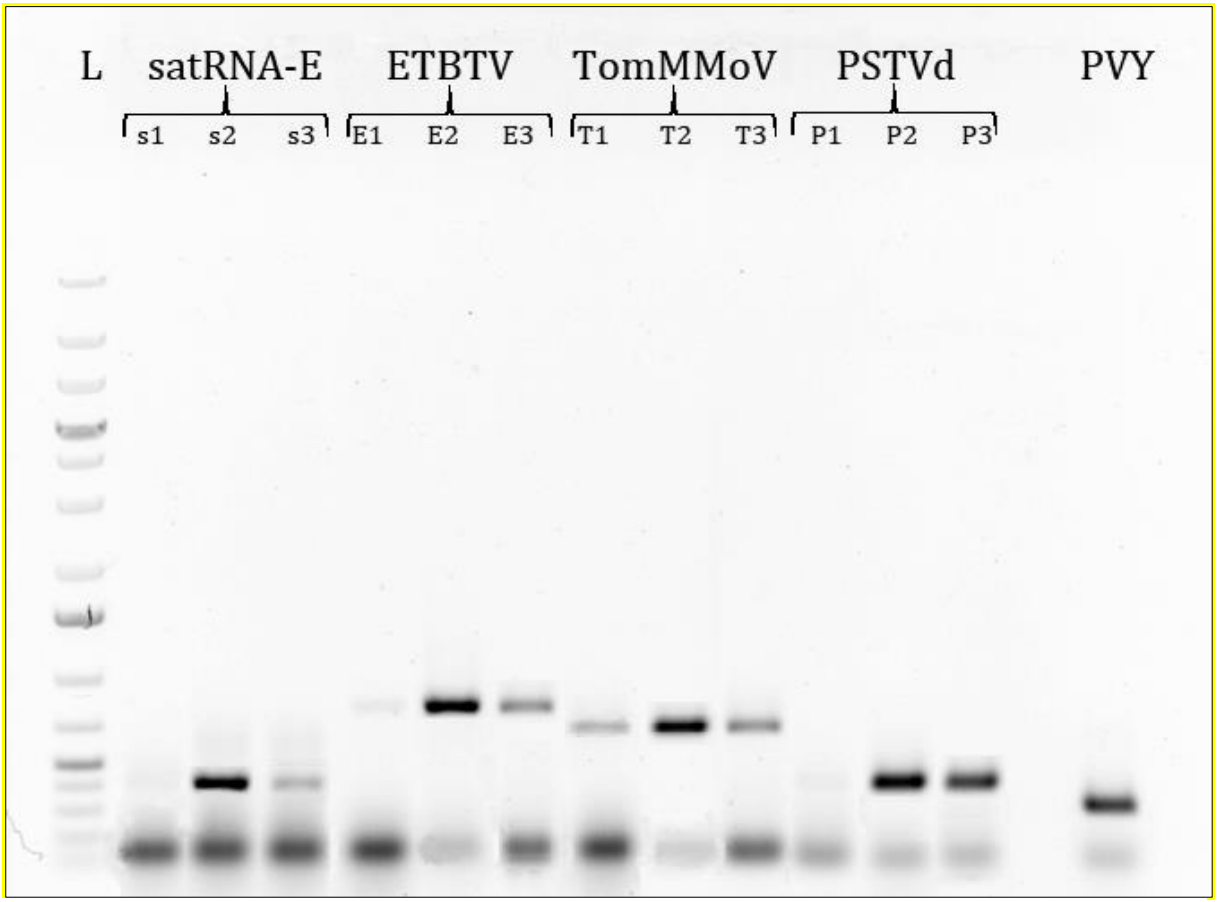


Figure 4.16 Gel image showing saturation of PCR product resulting from gradient PCR on three annealing temperatures for each virus. The ladder was a GeneRuler 1 kb Plus DNA Ladder^{TM}.

Tier one RT-PCR reactions indicated that PVY was the most prevalent of all the viruses with 12 out of 26 farms testing positive. Some of these farms which tested positive for PVY also tested positive for other viruses: farm 14 and 19 from Meru and Nandi respectively, tested positive for TMMoV; farm 8 and 13 from Embu and Meru, respectively tested positive for PSTVd; and lastly farm 11 from Tharaka Nithi tested positive for ETBTV and satRNA-E (Figure 4.17). All counties had at least one farm testing positive for one virus except for Elgeyo Marakwet which did not test positive for any of the viruses assayed using RT-PCR (Figure 4.18).

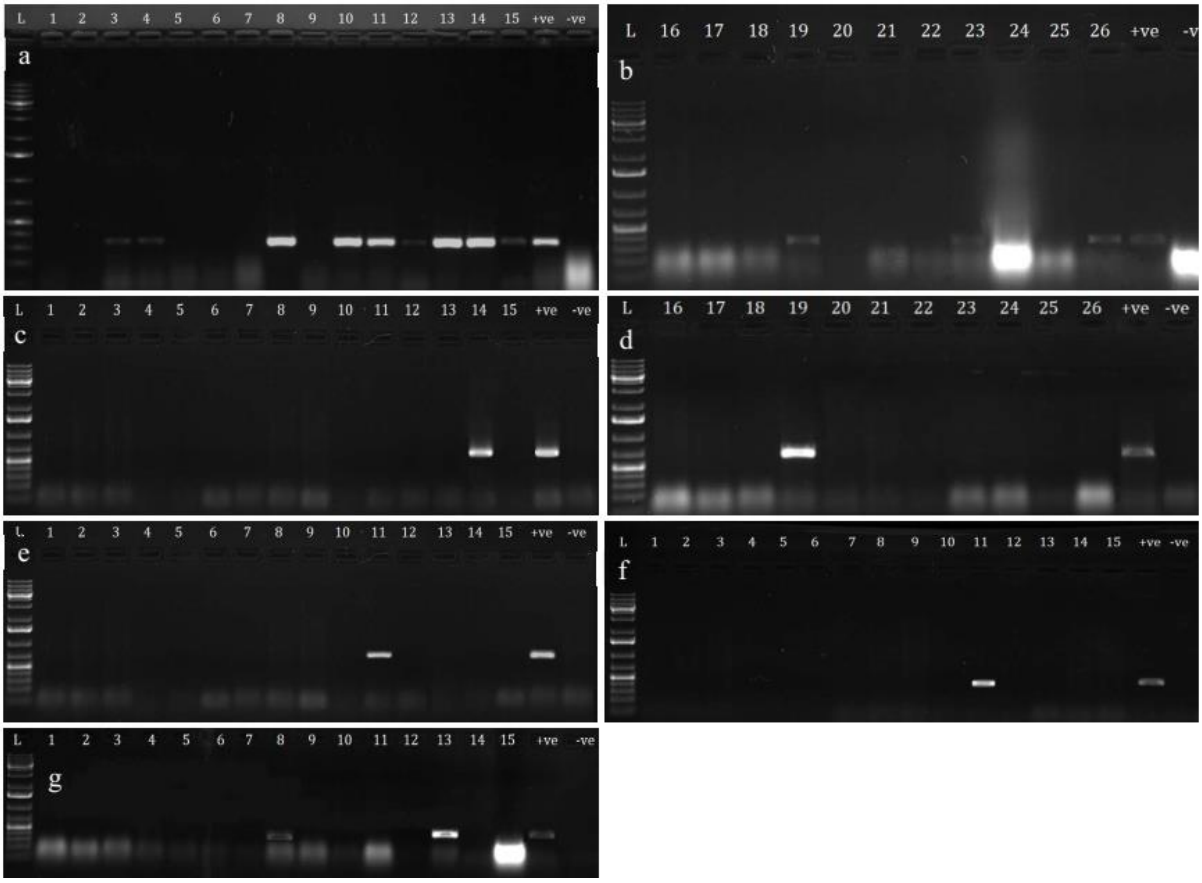


Figure 4.17 Tier one results showing gel electrophoresis results of RT-PCR reactions on 26 farms using primers listed in Table 4.8. Gels pictures are as follows: **a** and **b** are results for PVY, **c** and **d** are results for TMMoV, **e** and **f** are results for ETBTV and satRNA-E respectively, and **g** shows results for PSTVd. Farms were numbered 1 – 26.

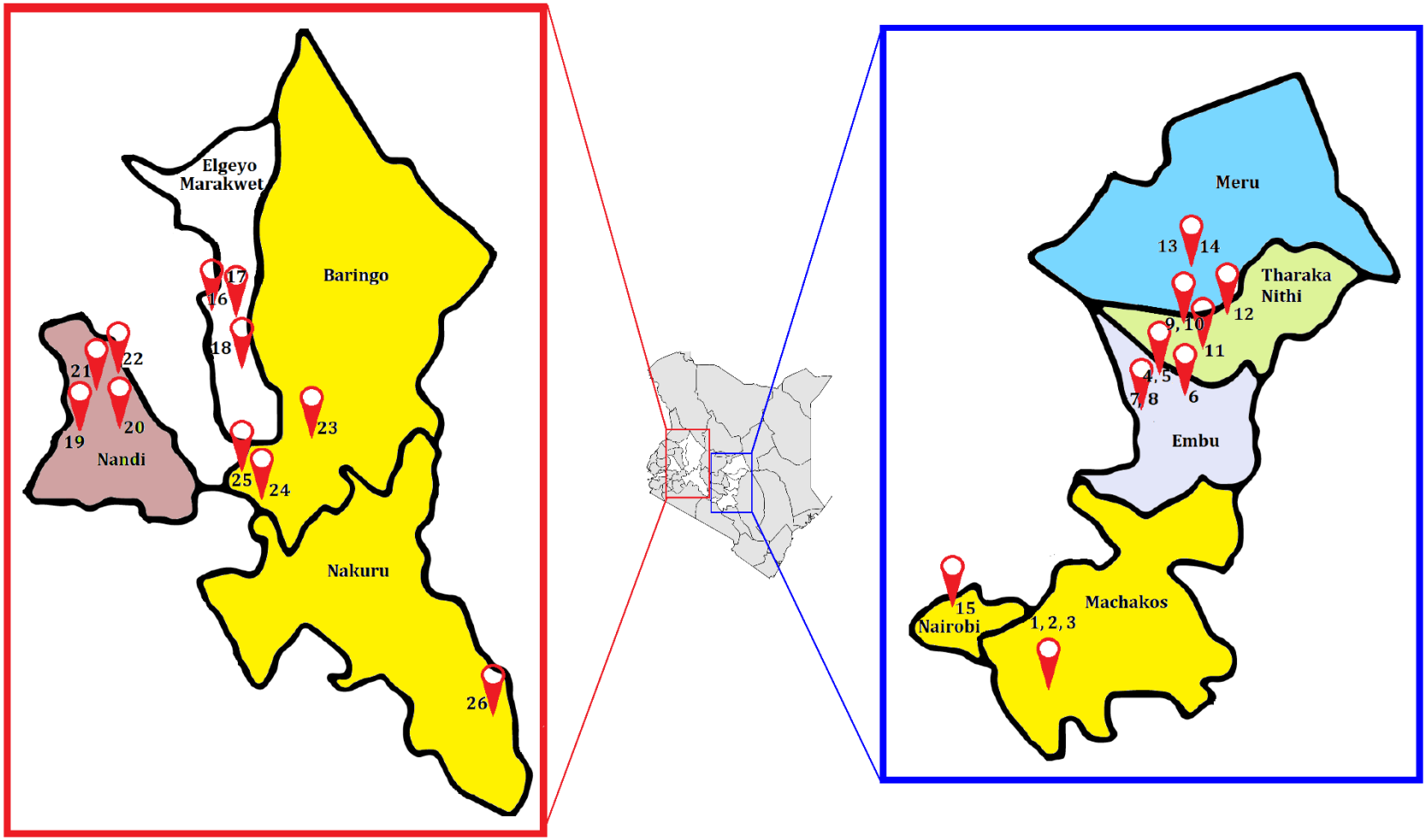


Figure 4.18 Map of the areas surveyed colour coded for the presence of a virus in the county as follows: yellow for potato virus Y (PVY) only; brown for PVY and tomato mild mottle virus (TMMoV); turquoise for PVY, TMMoV, and PSTVd; green for PVY and potato spindle tuber viroid (PSTVd); grey represents counties which tested for PVY, and ETBTV and satETBTV-E; and white for those that did not test positive for any of the viruses tested.

In tier two reactions, 72 samples tested positive for PVY: 36 samples from farms which had single infections; and another 36 samples from farms which had tested positive for two viruses; occurred in mixed infection of with either PSTVd, ETBTV and satETBTV-E, or TMMoV (Table 4.9). No sample had a single infection of PSTVd, ETBTV and satETBTV-E, or TMMoV. Testing individual samples for PVY showed it having an average farm incidence of 54.5%. The average incidence of PSTVd, and TMMoV was 51% and 60%, while ETBTV and satETBTV-E had an incidence of 54.5%.

A TTEST revealed there was no significant difference (P=0.01) in the means of PVY incidence between midland and lower highland regions (Table 4.9). No statistical analysis was applied for the other viruses as they only appeared in one or two farms: TMMoV two farms both of which fell under LH zones; PSTVd appeared in two farms, one in ML and the other in LH; and ETBTV and satETBTV-E appeared in ML. There was no farm from the upper highlands which was positive for any of the viruses tested for. The one farm in Baringo county that tested positive for PVY belonged to lower highland zone, while the other two belonged to upper highland zone (Table 4.3).

Table 4.9 A student's t-test analysis comparing the mean incidence of PVY in the midland and lower highland zones

County	Fields	Agro-ecological zone	No. of samples collected	Percentage incidence of PVY	Percentage incidence of the other viruses in mixed infection with PVY		
					PSTVd	ETBTV and satETBTV-E	TMMoV
Machakos	3	Lower highlands	14	50	-	-	-
Embu	4	Midlands	7	42.86	-	-	-
	8		7	71.43	57.14	-	-
Tharaka Nithi	10	Midlands	18	50	-	-	-
	11		11	54.55	-	54.55	-
	12		12	41.67	-	-	-
Meru	13	Lower highlands	11	45.45	45.45	-	-
	14		10	70	-	-	70
Nairobi	15	Midlands	10	60	-	-	-
Nandi	19	Lower highlands	28	50	-	-	50
Baringo	23	Lower highlands	16	18.75	-	-	-
Nakuru	26	Midlands	2	100	-	-	-
Mean				54.56	51.3	54.55	60
Ttest (p>0.01)				0.26			

4.2.3 Sanger sequencing results

All RT-PCR products which were taken for sequencing, yielded results which were consonant with sequences obtained using NGS. These PCR products showed a 90 – 99 percentage identity with their NGS assembled counterparts (Table 4.10).

Table 4.10 percentage alignment of the sequences obtained from Sanger sequencing of RT-PCR products of samples which tested positive for the various viruses

Virus	Sanger/NGS	Percentage alignment
ETBTV	MW731689/MW883068	90.17%
satRNA-E	MW713580/MW713579	99.51%
PSTVd	MZ054165/MZ054164	98.05%
	MZ054166/MZ054164	98.33%
TMMoV	OL800711/MW537585	96.70%
	OL555720/MW537585	96.54%

Sequences obtained from representative PVY positive samples from the 12 farms confirmed the very high conservation of the CP region targeted by the primers reported by Przewodowska *et al.* (2015). Alignment of the RT-PCR products showed a percentage alignment of between 98 – 99% (Table 4.11) and had a -0.1283 Tajima D value.

Table 4.11 Percentage identity matrix of the Sanger sequences obtained from the representative PVY positive samples of each of the 11 farms which tested positive

Isolate Name	Percentage identity										
PVY_1											
PVY_2	99.09										
PVY_3	98.79	98.49									
PVY_4	99.09	98.79	98.49								
PVY_5	99.7	99.4	99.09	99.4							
PVY_6	99.7	99.4	99.09	99.4	100						
PVY_7	99.7	99.4	99.09	99.4	100	100					
PVY_8	99.7	99.4	99.09	99.4	100	100	100				
PVY_9	98.79	98.49	99.09	98.49	99.09	99.09	99.09	99.09			
PVY_10	99.4	99.09	99.4	99.09	99.7	99.7	99.7	99.7	99.09		
PVY_11	99.09	98.79	99.4	98.79	99.4	99.4	99.4	99.4	99.7	99.4	
PVY_12	98.49	98.19	98.79	98.79	98.79	98.79	98.79	98.79	99.7	98.79	99.4

A phylogenetic tree of the Kenyan tree tomato isolates was drawn and compared with 24 strains of PVY (Green *et al*., 2017). Strain N:O showed the closest association to the Kenyan Isolates (Figure 4.22).

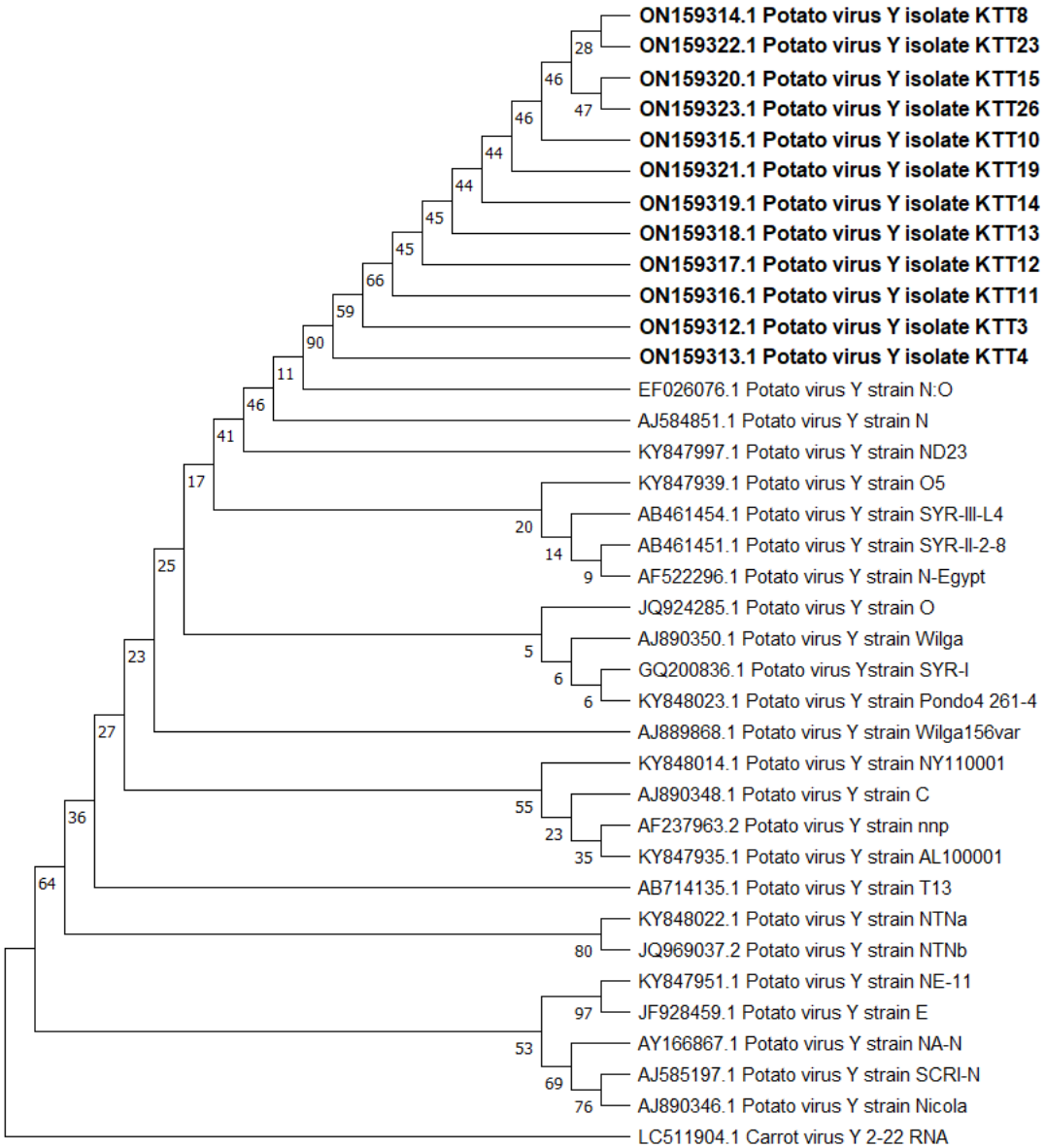


Figure 4.19 A neighbour-joining tree constructed with MEGA 11 using 1000 bootstrap replicates for isolates of PVY from various regions. Carrot virus Y was (NC 004013) was used as the outgroup. The Kenyan isolates, ON159312 - ON159323, are in bold.

CHAPTER 5 DISCUSSION

Viral diseases have been known to affect tree tomato in the field causing huge loses in cultivated land. This study was designed to identify, and to determine the occurrence and distribution of viruses infecting tree tomato in Kenya, whether in single or mixed infections. During the survey, virus associated symptoms including mosaics, mottling, stunting, leaf necrosis, vein banding and clearing, leaf curling and malformation, were all observed in different combinations. The observed symptoms were characteristic of many documented tree tomato virus infections (Ramírez-Gil *et al*., 2017), with mosaics, vein banding and leaf malformation being the most prevalent.

The lack of sufficient difference in the incidence of virus associated symptoms in the three agro-ecological zones suggests that altitude does not contribute to the spread of the viruses. There was a significantly higher severity in the midland and lower highlands which could suggest a positive correlation of virus diseases with the higher temperatures experienced in these zones. The higher severity in these two zones could also be as a result of a higher population of virus vectors which transmit viruses and higher temperatures ideal for virus replication and symptom expression. This is supported by the fact that no farm which fell in the upper highlands zones tested positive for any virus which was tested. Known virus vectors, aphids (for PVY) and whiteflies (for TMMoV, ETBTV and associated satellite), were also observed in some of the farms from Embu, Tharaka Nithi, Meru and Elgeyo Marakwet. Intercropping with crops like tobacco, potato, tomato and other members of the *Solanaceae* family and presence of weeds, both of which can act as virus reservoirs, also exposed tree tomato plants to a larger number of viruses which have previously not been known to infect the crop (Hančinský *et al*., 2020).

Other symptoms identified by farmers which are not necessarily virus associated included dieback, mildew, root rot and blight. These symptoms have been recorded in other research as

being caused by fungal species (Ramírez-Gil *et al*., 2017). These other symptoms could also hinder proper diagnosis by symptomatology. The lack of information by the farmers on seed certification, recycling, free movement of potentially infected material from place to place, lack of a tested method to clean up the infected high yielding materials in the field, and planting new orchards near the old ones, unsanitary agronomic practices such as rouging, possibly play a role in the spread of viruses and thus high incidence which was observed in this study. Using NGS this study confirmed the presence of PVY and TMMoV, and revealed the presence of PSTVd, ETBTV and satETBTV-E infecting tree tomato in the country.

Potato virus Y was first identified to cause disease in tree tomato by Mbula (1992) using the physical properties, thermal inactivation point, dilution end point and longevity in vitro. Physical properties were highly unspecific. The identity of the PVY strain infecting tree tomato in Kenya at this moment was not identified. This study was also not able to classify any of the reads to a particular known strain or recombinant strain. The genome of PVY is very diverse and so far there are five known parent strains and 19 known recombinants (Green *et al*., 2017). A PVY genome was thus not established. The mapping of the NGS reads signified that fragments of O, C, NA-N and Eu-N present, which could be an indication that either they are present or recombinant strains composed of fragments of said strains was infecting tree tomato in Kenya. For a proper strains' identification of the different PVY isolates, individual samples have to be sequenced to produce full genomes, serology done and symptomatology in potato done (Green *et al*., 2017; Glasa *et al*., 2021).

Tomato mild mottle virus was first detected in tree tomato in 2010 and a partial coat protein sequence deposited to GenBank (accession HQ711860). The polyprotein sequence was fairly conserved against both strains as both showed selection that constrained the non-synonymous changes. Because the genetic code is degenerate, the observed ~20% difference in the

nucleotide sequence between the two strains only equates to a ~10% difference in the amino acid sequence. Apart from the ~20% difference in the sequence similarity between the two known strains, the other difference is on the ability to elicit symptoms of disease in tomato where TMMoV-E can while TMMoV-I cannot (Dombrovsky *et al*., 2013). Though the Kenyan isolate has not been tested on whether it can elicit symptoms in tomato, genetic similarity point toward it belonging to TMMoV-E.

The different proteins are predicted to function as follows: P1 and Helper component (HC) protein as a Protease and RNA silencing suppressor (RSS); cylindrical inclusion (CI) Cytoplasmic inclusion bodies, cell-to-cell movement and as a helicase; viral protein genome-linked (Vpg) in protection of the 5′ region; nuclear inclusion protein a (Nia) as nuclear inclusion protease; nuclear inclusion protein b (Nib) as a nuclear inclusion polymerase; and the coat protein (CP) in genome encapsidation and long-distance movement (Dombrovsky *et al*., 2014). The functions of P3 protein, PIPO, 6K1 and 6K2 remain unknown for Ipomoviruses.

Ethiopian tobacco bushy top virus was first discovered in Ethiopia in 2014 infecting tobacco along with its associated RNA satellite (satRNA-E; Abraham *et al.* 2014). It has also been detected in Malawi and Zambia, infecting tobacco and beans, respectively (Mulenga *et al*., 2020; Udagawa *et al*., 2020). The virus does not encode for any structural proteins and its RNA is usually encapsidated by helper viruses. The exact function of the satellite RNA in ETBTV has not yet been identified. The associated RNA satellite of groundnut rosette virus (GRV) is the actual cause of disease symptoms in groundnut and is crucial in facilitating polerovirus-dependent aphid transmission (Wangai and Lelgut, 2006; Ryabov, 2012).

In Ethiopia, ETBTV along with its RNA satellite was found to be transmitted alongside potato leafroll virus while in Zambia it was found to be transmitted alongside cowpea polerovirus 1, both members of the *Polerovirus* genus. In both cases, the two *Polerovirus* are thought to act

as helper. *Umbraviruses* have so far been found to depend on assistor viruses from the family *Solemoviridae* for encapsidation and thus transmission (Taliansky and Ryabov, 2008). In cases where single infections of members of the *Umbravirus* genus have been found to occur, it is conjectured that the infective RNA is protected by lipid-containing structures or other plant cell derived components (Ryabov, 2012). The only member of *Solemoviridae* family detected using NGS was pepper enamovirus (PeEV). Work is ongoing to confirm the presence of PeEV in the samples using RT-PCR.

Potato spindle tuber viroid is a small single stranded circular RNA and is the type species for the genus *Pospiviroids*. It is mostly found infecting solanaceous plants but has also been known to infect avocado, citrus and other cosmetic plants (Ryabov, 2012). The viroid has been known to reduce yield in susceptible potato varieties by up to 70% (Annenkov, 2000). Viroid replication is very prone to errors and can lead to the formation of quasi species (mutant species derived from the parent RNA) which can make up to 30% of the of the RNA viroid population in 14 days after inoculation (Adkar-Purushothama *et al*., 2020). Transmission experiments of PSTVd by Singh (1973) did not produce any symptoms in tree tomato. There were no attempts to identify or differentiate quasi species from the sequences which aligned to PSTVd. This was the first report of a natural infection of tree tomato by PSTVd.

Five other viruses were detected using NGS but have not been characterised nor their presence confirmed using RT-PCR. Two of them, Kenyan potato cytohabdovirus and tamarillo fruit ring virus have only recently been discovered as new viruses and their sequences were not available in the GenBank virus database during the initial analysis that revealed the other viruses.

In order to confirm the presence of viruses detected using NGS, primers were developed using identified sequences for PCR analysis. All the primers were able to detect their specific viruses with high precision. Of all the viruses, PVY had the highest prevalence, testing positive in 46%

of the farms sampled. The high percentage identity and the negative Tajima D value of the CP sequences, were indicative of a high degree of sequence conservation both at the nucleotide and amino acid level of this region. Because of this, and the inadequacy of the sequences of the target region alone in revealing the strain or recombinant strains, the inferred phylogenetic association between strains N:O and the Kenyan isolates could be coincidental. The lack of evidence of recombination on the fourth RJ could be as a result of the fact recombination at the RJ4 is not common among the known PVY recombinants. The primers used in this study are ideal for the indiscriminate detection of PVY strains.

In both farms 14 and 19, all samples which tested positive for TMMoV also tested positive for PVY. Symptoms observed in this farm included mottling, and leaf malformation. While PVY is transmitted by aphids, there is a contention on whether TMMoV is transmitted by aphids or whiteflies. Two studies, Walkey *et al.* (1994) and Hiskias *et al*. (2001), reported transmission by aphids. Another two, Abraham *et al.* (2012) and Dombrovsky *et al.* (2013), reported whitefly transmission. There is a conjecture that like other members of the genus *Ipomovirus*, the natural vector of TMMoV are whiteflies, and that cases where aphid transmitssion was reported, it was in mixed infection with PVY (Dombrovsky, *et al.*, 2014). This could explain why TMMoV occurred in mixed infection with PVY in all cases in this study and in some samples in a similar study on tomato in Ethiopia (Hiskias *et al.*, 1999). Cases of mixed infection of PVY and TMMoV were also detected in tomato in Ethiopia, and were found to elicit severe mosaics, leaf malformation and stunting, which suggested synergy of the two.

Similarly, in the samples which tested positive for PSTVd, all were found to have a mixed infection with PVY. The transmission of PSTVd is mostly by mechanical means. Although cases of aphid transmission of PSTVd are rare, transmission experiments by *Myzus euphorbiae* were found to be successful (Bokx and Piron, 1981). In a different study, transmission of

PSTVd was heterologously encapsidated into the PLRV particle, protecting the RNA and thus facilitating its spread (Querci *et al*., 1997). The co-infection of samples with PSTVd and PVY in both farms 8 and 13 in from Embu and Meru, respectively, could suggests a similar situation. Indeed, a similar case of co-infection of PVY and PSTVd on potato plants has been reported previously in Russia (Romanova, 2007). The co-infection of the two could also be as a result of simultaneous transmission from other reservoir hosts such as potatoes. This is supported by the fact that both regions where PSTVd was detected, Embu and Meru, are potato growing regions, making interspecies transmission very likely. The symptoms observed in these farms included chlorosis and leaf malformation. There was no symptom difference between the samples which had a mixed infection of PSTVd and PVY, and a single infection of PVY, suggesting no synergy. The experimental host range of PSTVd includes tree tomato where it was found to produce no symptoms (Singh, 1973). This could suggest that the symptoms which were observed were as a result of PVY only.

ETBTV and satRNA-E also occurred in mixed infection with PVY. As indicated above, ETBTV does not encode for any structural proteins, and has been found encapsidated by members of the polerovirus and enamovirus genus which aid in its transmission. Although PeEV was detected via NGS, its presence is yet to be validated by PCR in samples which tested positive for ETBTV and PVY. The samples which tested positive for tree ETBTV, satRNA-E and PVY exhibited symptoms of leaf curling, malformation and vein clearing. These symptoms could however not be attributed to any of the above viruses or as a result of synergism. Research is ongoing to establish the exact symptoms which would be exhibited in single infections of ETBTV and mixed infection with PVY.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Using NGS for virus identification, this study was able to identify the presence of three viruses, PVY, TMMoV, ETBTV, one viroid, PSTVd, and an RNA satellite and satRNA-E, infecting tree tomato in nine counties in Kenya, characterised their genomes and deduced their incidence and distribution. Other viruses detected using NGS but not characterized in the study were pepper enamovirus, tobacco mottle virus, tomato chlorosis virus Kenyan potato cytohabdovirus and tamarillo fruit ring virus.

Using RT-PCR and sanger sequencing as a confirmatory independent technique the presence of these viruses was confirmed. The RT-PCR reactions also revealed PVY to be having the highest prevalence while TMMoV, PSTVd, ETBTV and satRNA-E, were found to be present in one or two farms and all occurred in mixed infection with the other PVY. Using data from this study, mitigation measures can be started so as to prevent further spread of these viruses to other orchards, as well as to other susceptible crops.

6.2 Recommendations

- Further studies need to be done to confirm the presence of the other unconfirmed viruses – pepper enamovirus, tobacco mottle virus, tomato chlorosis virus, Kenyan potato cytohabdovirus, and tamarillo fruit ring virus – using PCR.
- The full genomes of PVY isolates infecting tree tomato need to be determined in order to properly characterise and group into specific strains and recombinant.
- Transmission studies for TMMoV and ETBTV and satETBTV-E need to be done so as to characterise disease symptoms of single infection of in tree tomato.
- The association of TMMoV and PVY need to be established and confirm whether the later acts as a helper virus that facilitate the former's aphid transmission.

- Proper weeding and rouging should be done to ensure elimination of potential virus reservoirs or inoculum source are present in the farm.
- Control of aphids and whiteflies should be done so as to curb spread of viruses through vectors.
- Clean seed programs need to be established to ensure that farming starts virus free plants.

REFERENCES

Abdul Kadir, N. A., Rahmat, A., & Jaafar, H. Z. (2015). Protective effects of tamarillo (Cyphomandra betacea) extract against high fat diet induced obesity in sprague-dawley rats. *Journal of Obesity, 2015*(846041), 1–8. https://doi.org/10.1155/2015/846041

Abdul Mutalib, M., Rahmat, A., Ali F, O. F., & Ramasamy, R. (2017). Nutritional compositions and antiproliferative activities of different solvent fractions from ethanol extract of Cyphomandra betacea (tamarillo) fruit. *Malaysian Journal of Medical Sciences, 24*(5), 19–32. https://doi.org/10.21315/mjms2017.24.5.3

Abraham, A., Menzel, W., Vetten, H., & Winter, S. (2012). Analysis of the tomato mild mottle virus genome indicates that it is the most divergent member of the genus Ipomovirus (family Potyviridae). *Archives of Virology, 157*, 353–357. https://doi.org/https://doi.org/10.1007/s00705-011-1167-9

Adams, I., Miano, D., Kinyua, Z., Wangai, A., Kimani, E., Phiri, N., Reeder, R., Harju, V., Glover, R., Hany, U., Souza-Richards, R., Deb Nath, P., Nixon, T., Fox, A., Barnes, A., Smith, J., Skelton, A., Thwaites, R., Mumford, R., & Boonham, N. (2013). Use of next-generation sequencing for the identification and characterization of Maize chlorotic mottle virus and Sugarcane mosaic virus causing maize lethal necrosis in Kenya. *Plant Pathology, 62*(4), 741-749. https://doi.org/https://doi.org/10.1111/j.1365-3059.2012.02690.x

Adkar-Purushothama, C., Bolduc, F., Bru, P., & Perreault, J.-P. (2020). Insights into Potato spindle tuber viroid quasi-species from infection to disease. *Frontiers in Microbiology, 11*, 1235. https://doi.org/https://doi.org/10.3389/fmicb.2020.01235

Amaro, A., Petretto, A., Angelini, G., & Pfeffer, U. (2016). Advancements in omics sciences. In A. Shahzad (Ed.), *Translational Medicine: Tools and Techniques* (pp. 67–108). Elsevier Inc.

Ames, T., Smit, N., Braun, A., O'Sullivan, J., & Skoglund, L. (1996). *Sweetpotato: Major Pests, Diseases, and Nutritional Disorders.* Peru: International Potato Center.

Annenkov, B. (2000). Problems and prospects of the influence of curative measures on the potato industry performance in the Russian far east. In *Topical Issues of Crop Immunity, Sanitation, and Protection in the Russian Amur Region* (pp. 26-42). Khabarovsk.

Atkinson, R., & Gardner, R. (1993). Regeneration of transgenic tamarillo plants. *Plant Cell Reports, 12*(6), 347-351. https://doi.org/10.1007/bf00237433

Baranwal, V., Kapoor, R., S., K., & Srivastava, N. (2020). Recent advances of virus diagnostics in horticultural crops. In Awasthi, *Applied Plant Virology: Advances, Detection, and Antiviral Strategies* (pp. 27-33). Academic Press.

Bioversity International. (2013). *Descriptors for tree tomato (Solanum betaceum Cav.) and wild relatives.* Retrieved from http://www.bioversityinternational.org

Bokx, J., & Piron, P. (1981). Transmission of Potato spindle tuber viroid by aphids. *Netherlands Journal of Plant Pathology, 87*(2), 31–34. https://doi.org/10.1007/bf01976653

CABI. (2021). *Potato spindle tuber viroid*. Retrieved 04 30, 2021, from Invasive Species Compendium: https://www.cabi.org/isc/datasheet/43659

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. (2009). BLAST+: architecture and applications. *BMC Bioinformatics, 10*(421).

Cornejo-Franco, J., Alvarez-Quinto, R., Grinstead, S., Mollov, D., Karasev, A., Ochoa, J., & Quito-Avila, D. (2019). A new Tymovirus isolated from Solanum quitoense: characterization and prevalence in two solanaceous crops in Ecuador. *Plant Disease, 103*, 2246-2251. https://doi.org/https://doi.org/10.1094/PDIS-01-19-0113-RE

Dombrovsky, A., Reingold, V., & Antignus, Y. (2014). Ipomovirus – an atypical genus in the family Potyviridae transmitted by whiteflies. *Pest Management Science, 70*(10), 1553–1567. https://doi.org/10.1002/ps.3735

Dombrovsky, A., Sapkota, R., Lachman, O., & Antignus, Y. (2011). Eggplant mild leaf mottle virus (EMLMV), a new putative member of the genus Ipomovirus that harbors an HC-Pro gene. *Virus Genes, 44*(2), 329–337. https://doi.org/10.1007/s11262-011-0686-5

Dombrovsky, A., Sapkota, R., Lachman, O., Pearlsman, M., & Antignus, Y. (2013). A new aubergine disease caused by a whitefly-borne strain of Tomato mild mottle virus (TomMMoV). *Plant Pathology, 62*, 750-759. https://doi.org/10.1111/ppa.12004

Eagles, R., Gardner, R., Richard, L. S., & Forster, R. (1994). Incidence and distribution of six viruses infecting tamarillo (Cyphomandra betacea) in New Zealand,. *New Zealand Journal of Crop and Horticultural Science,, 22*(4), 453-458. https://doi.org/10.1080/01140671.1994.9513857

Edwards, S. (2014). *Cross protection and molecular characterisation of three Tamarillo mosaic virus isolates.* MSc Thesis: The University of Auckland.

EFSA PLH Panel. (2013). Scientific opinion on the risk to plant health posed by Arabis mosaic virus, Raspberry ringspot virus, Strawberry latent ringspot virus and Tomato black ring virus to the EU territory with the identification and evaluation of risk reduction options. *EFSA Journal, 11*(10), 3377. https://doi.org/10.2903/j.efsa.2013.3377

FAO. (2005). *Irrigation in Africa in figures.* AQUASTAT Survey.

Francki, R. (1980). Limited value of the thermal inactivation point, longevity in vitro and dilution endpoint as criteria for the characterization, identification and classification of plant viruses. *Intervirology, 13*(2), 91-98. https://doi.org/https://doi.org/10.1159/000149113

Fredricks, D., & Relman, D. (1996). Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clinical microbiology reviews, 9*(1), 18–33. https://doi.org/https://doi.org/10.1128/CMR.9.1.18

Glasa, M., Hančinský, R., Šoltys, K., Predajňa, L., Tomašechová, J., Hauptvogel, P., Mrkvová, M., Mihálik, D., & Candresse, T. (2021). Molecular characterization of Potato Virus Y (PVY) using high-throughput sequencing: constraints on full genome reconstructions imposed by mixed infection involving recombinant PVY strains. *Plants, 10*(4), 753. https://doi.org/https://doi.org/10.3390/plants10040753

Green, K., Brown, C., Gray, S., & Karasev, A. (2017). Phylogenetic study of recombinant strains of Potato virus Y. *Virology, 507*, 40-52. https://doi.org/https://doi.org/10.1016/j.virol.2017.03.018

Gutierrez, P., Alzate, J., & Montoya, M. (2015). Genome sequence of a virus isolate from tamarillo(Solanum betaceum) in Colombia: evidence for a new potyvirus. *Archives of Virology, 160*(2), 557–560. https://doi.org/10.1007/s00705-014-2296-8

Hančinský, R., Mihálik, D., Mrkvová, M., Candresse, T., & Glasa, M. (2020). Plant Viruses Infecting Solanaceae Family Members in the Cultivated and Wild Environments. *A Review. Plants, 9*(5), 667. https://doi.org/https://doi.org/10.3390/plants9050667

HCD. (2017). *Horticulture validated report 2016-2017.* Horticultural Crops Directorate, Nairobi.

HCD. (2018). *Horticulture validated report 2017-2018.* Agriculture and Food Authority.

HCD. (2020). *Horticulture validated report 2019-2020.* The Horticultural Crops Directorate.

HCDA. (2008). *Horticulture data 2005-2007 validation report.* Nairobi: Horticultural Crops Development Authority.

Hiskias, Y., Lesemann, D., & Vetten, H. (1999). Occurrence, distribution and relative importance of viruses infecting hot pepper and tomato in the major growing areas of Ethiopia. *Journal of Phytopathology, 147*(1), 5–11. https://doi.org/10.1111/j.1365-3059.1994.tb01638.x

Hiskias, Y., Lesemann, D., & Vetten, H. (2001). Biological characteristics of tomato mild mottle virus a potyvirus isolated from tomato. *African Crop Science Journal, 9*(3), 517-525. https://doi.org/10.4314/acsj.v9i3.27598

Insuasti, M., Ochoa, J., Martin, R., Alvarez, R., & Quito-Avila, D. (2016, April). First Report of Potato virus V and Peru tomato mosaic virus on Tamarillo (Solanum betaceum) Orchards of Ecuador. (A. Robertson, Ed.) *Plant Disease, 100*(4), 868. https://doi.org/dx.doi.org/10.1094/PDIS-09-15-1063-PDN

Jaramillo, M., Gutiérrez, P., Lagos, L., Cotes, J., & Marín, M. (2011). Detection of a complex of viruses in tamarillo (Solanum betaceum) orchards in the Andean region of Colombia. (M. Zerbini, Ed.) *Tropical Plant Pathology, 36*(3), 150-159.

Kaiser, W. (1980). Use of thermotherapy to free potato tubers of Alfalfa mosaic, Potato leaf roll, and Tomato black ring viruses. *Phytopathology, 70*(11), 1119-1122.

Kamau, P. (2014, January). Earn more from tree tomato production. (C. Nyakundi, & P. Kamau, Eds.) *The Organic Farmer*, p. 3. Retrieved from http://www.infonet-biovision.org/res/res/files/4307.TOF%20January%202014red.pdf

Kanali, C., Kituu, G., Mutwiwa, U., Mung'atu, J., Ronoh, E., Njuguna, S., Kamwere, M., & Mulamu, L. (2017). *Energy efficient rural food processing utilizing renewable energy to improve rural livelihoods in Kenya.* Project, Jomo Kenyatta University of Agriculture and Technology, Nairobi.

Kilavi, M. (2012, 07 11). *Kenya's Climate.* Retrieved 07 06, 2017, from https://www.liverpool.ac.uk/media/livacuk/qweci/QWeCI,science,talk,Mary,Kilavi.pdf

Kochetov, A., Pronozin, A., Shatskaya, N., Afonnikov, D., & Afanasenko, O. (2021). Potato spindle tuber viroid. *Vavilovskii zhurnal genetiki i selektsii, 25*(3), 269-275. https://doi.org/doi:10.18699/VJ21.030

Macharia, I., Backhouse, D., Skilton, R., Ateka, E., Wu, S.-B., Njahira, M., & Harvey, J. (2015). Diversity of Thrips Species and Vectors of Tomato Spotted Wilt Virus in Tomato Production Systems in Kenya. *Journal of Economic Entomology, 108*(1), 20-28. https://doi.org/https://doi.org/10.1093/jee/tou010

Mbula, B. B. (1992). *Potato virus Y (PVY) in irish potatoes (Solanum tuberosum) and tree tomato (Cyphomandra betaceae) and the influence of potato susceptibility to the virus and the aphid virus-vector on the spread of PVY Mosaic in Kenya (PhD).* Nairobi: University of Nairobi.

Mejía, D., Rodas, E., Patiño, L., & González, E. (2009). Effect of acibenzolar-s-methyl on virus infection progress caused by potyvirus in tree tomato. *Agronomia Colombiana, 27*(1), 87-93.

Monger, W., & Nixon, T. (2010). *Tomato mild mottle virus in Tamarillo in Kenya.* Novel Methods, Food and Environment Reseach Agency.

Morton, J. (1987). *Tree Tomato.* Miami, FL.: Julia F. Morton.

Morton, J. M. (1982). *The tree tomato, or "tamarillo", a fast-growing, early-fruiting small tree for subtropical climates* (Vol. 95). Miami, FL: Julia F. Morton.

Mulenga, R., Miano, D., Kaimoyo, E., Akello, J. M., Simulundu, E., & Alabi, O. (2020). First report of Ethiopian tobacco bushy top virus and its associated satellite RNA infecting common bean (Phaseolus vulgaris). *Plant Disease, 105*(2), 516.

Muriithi, C., Matiri, F., Kihanda, F., Maina, D., & Kasungo, F. (2013). Evaluation of potential of tree tomato production and marketing in eastern Kenya for improved nutrition and food securit. 27th Soil Science Society of East Africa and the 6th African Soil Science Society.

Ochoa, J., & Tolin, S. (2012). Virus identifcation in tree tomato in Ecuador. *Toward the effective integrated pest management of plant diseases caused by viruses in developing countries* (pp. 105-106). India: InternatIonal Plant vIrus DIsease network (IPvDn).

Opiyo, S., Ateka, E., Owuor, P., Manguro, L., & Karuri, H. (2010). Survey of sweet potato viruses in western Kenya and detection of Cucumber mosaic virus. *Journal of Plant Pathology, 92*(3), 797-801.

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., & Anthony, S. (2009). *Agroforestree Database: a tree reference and selection guide version 4.0* . Retrieved October 16, 2016, from World Agroforestry Centre: http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp

Otieno, E. (1985). *Identification of tomato mosaic strain of tobacco mosaic virus (TMV) and its effects on yield of tomato (Lycopersicon esculentum) valueties moneymakei and Roma V F in Kenya.* Nairobi: University of Nairobi Upper Kabete Library.

Pliansinchai, U., & Teakle, D. (1994). Susceptibility of tamarillo to seven plant viruses. *Australasian Plant Pathology, 23,* 25–28.

Prohens, J., & Nuez, F. (2001). The Tamarillo (Cyphomandra betacea): A Review of a promising small fruit crop. *Small Fruits Review, 1*(2), 43-68. https://doi.org/10.1300/J301v01n02_06

Przewodowska, A., Zacharzewska, B., Chołuj, J., & Treder, K. (2015). A one-step, real-time reverse transcription loopmediated isothermal amplification assay to detect Potato Virus Y. *American Journal of Potato Research, 92*(3), 303–311. https://doi.org/10.1007/s12230-015-9430-3

Querci, M., Owens, R., Bartolini, I., Lazarte, V., & Salazar, L. (1997). Evidence for heterologous encapsidation of potato spindle tuber viroid in particles of potato leafroll virus. *Journal of General Virology, 78*(6), 1207-1211. https://doi.org/10.1099/0022-1317-78-6-1207

Quezada, P. (2011). *Caracterización morfológica y molecular de tomate de árbol, solanum betaceum cav. (solanaceae).* Madrid: Universidad Politécnica de Madrid.

Ramírez-Gil, J., Gil-Aguirre, A., & Morales-Osorio, J. (2017). Etiology of tree tomato (Solanum betaceum CAV.) diseases. *Revista de Protección Vegetal, 32*(1), 33-51.

Riang'a, R., Broerse, J., & Nangulu, A. (2017). Food beliefs and practices among the Kalenjin pregnant women in rural Uasin Gishu County, Kenya. *Journal of Ethnobiology and Ethnomedicine, 13*(29), 1-16. https://doi.org/10.1186/s13002-017-0157-8

Romanova, S., Volkov, Y., Kakareka, N., Pleshakova, T., & Kozlovskaya, Z. (2007). Potato disease caused by combined infection with potato spindle tuber viroid and Potato virus Y necrotic strain. *Russian Agricultural Sciences, 33*(3), 162–165. https://doi.org/10.3103/S1068367407030081

Ryabov, E. T. (2012). Part II – The positive sense single stranded RNA viruses: Umbravirus. In A. King, E. Lefkowitz, & M. Adams (Eds.), *Virus Taxonomy: The Classification and Nomenclature of Viruses The 9th Report of the ICTV* (pp. 1191-1195). London: Elsevier Inc.

Sale, P. (1983). Tamarillos pests and diseases insects, nematodes, fungi, bacteria, virus, symptoms and biology. *New Zealand Ministry of Agriculture and Fisheries AgLink, 298*, 1-4.

Schotsmans, W. C., East, A., & Woolf, A. (2011). Tamarillo (Solanum betaceum (Cav.)). Postharvest Biology and Technology of Tropical and Subtropical Fruits . In E. M. Yahia, *Postharvest Biology and Technology of Tropical and Subtropical Fruits* (Vol. Volume 4 : Mangosteen to white sapote, pp. 427–442e). https://doi.org/10.1533/9780857092618.427

Singh, R. P. (1973). Experimental host range of the potato spindle tuber "virus.", 50(4), 111–123. *American Potato Journal*. https://doi.org/https://doi.org/10.1007/BF02857207

Sombroek, W., Braun, H. M., Pouw, B. J., & Survey, K. S. (1982). *Exploratory Soil Map and Agro-climatic Zone Map of Kenya, 1980: Scale 1:1,000,000.* Kenya Soil Survey.

SPHDS. (2012). *Diagnostic protocol for the identification and detection of Candidatus Liberibacter solanacearum, the causal agent of zebra chip of potatoes.* Subcommittee on Plant Health Diagnostic Standards.

Ssekyewa, C. (2006). *Incidence, distribution and characteristics of major Tomato leaf curl and mosaic virus diseases in Uganda* (Vols. PhD-thesis.). Ghent: Faculty of Bioscience Engineering, Ghent University,.

Stevens, W. A. (1983). *Virology of flowering plants.* New York: Springer Science+Business Media.

Taliansky, M., & Ryabov, E. (2008). Umbravirus. In B. Mahy, & M. Van Regenmortel (Eds.), *Encyclopedia of Virology* (3rd ed., pp. 209-213). Academic Press. https://doi.org/https://doi.org/10.1016/B978-012374410-4.00524-0

Udagawa, H., Koga, K., Shinjo, A., Kitashiba, H., & Takakura, Y. (2020). Reduced susceptibility to a tobacco bushy top virus Malawi isolate by loss of function in host eIF(iso) 4E genes. *Breeding Science, 70*(3), 313-320. https://doi.org/https://doi.org/10.1270/jsbbs.19135

Walkey, D., Spence, N., Clay, C. M., & Miller, A. (1994). A potyvirus isolated from solanaceous hosts. *Plant Pathology, 43*(5), 931–937. https://doi.org/doi:10.1111/j.1365-3059.1994.tb01638.x

Wangai, A., & Lelgut, D. (2006). Status of potato viruses in Africa. *Plant Disease, 80*(83), 458-465.

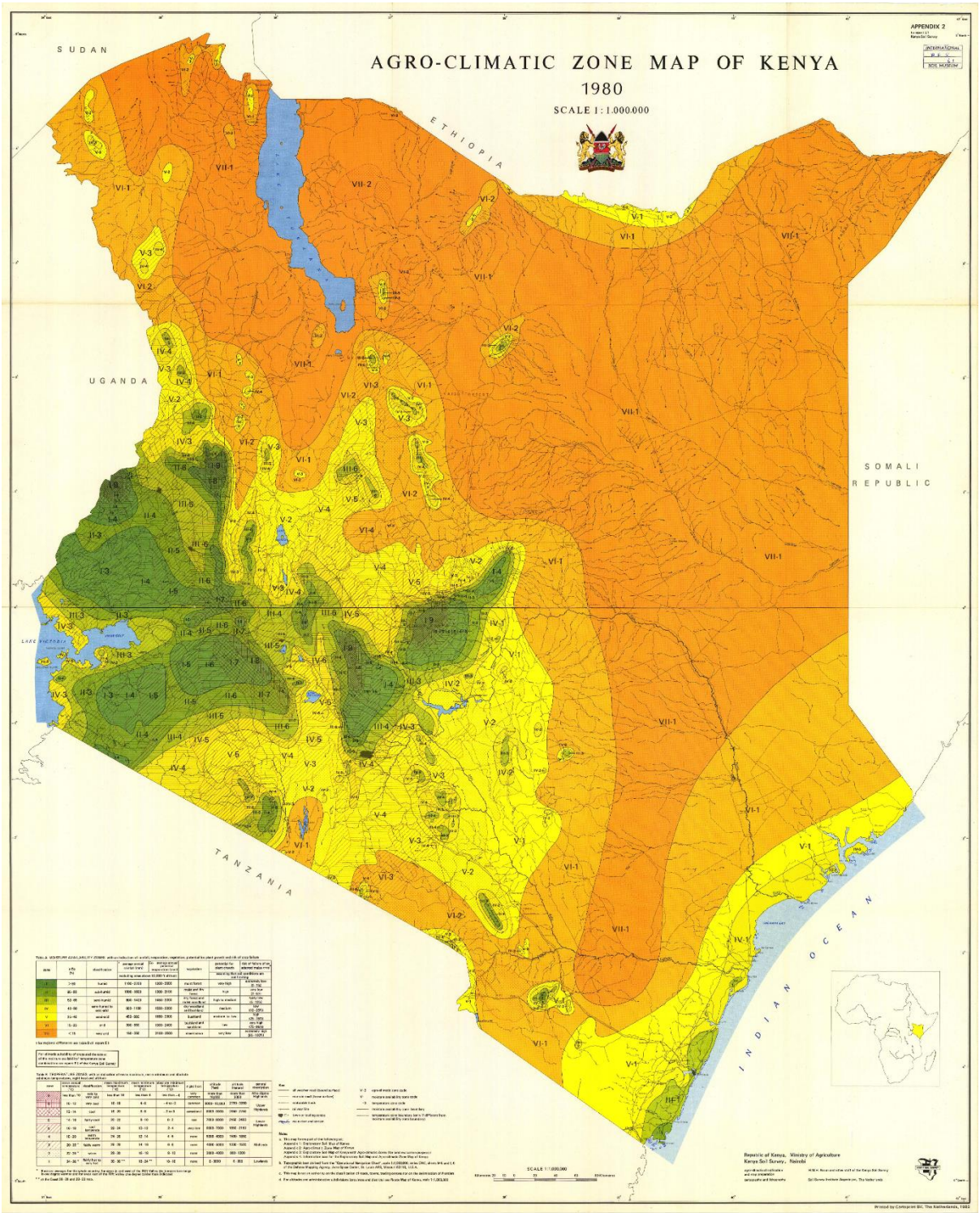
Wangai, A., Mandal, B., Pappu, H., & Kilonzo, S. (2001). Outbreak of Tomato spotted wilt virus in tomato in Kenya. *Plant Disease, 85*(10), 1123. https://doi.org/dx.doi.org/10.1094/PDIS.2001.85.10.1123B

Were, H., Kabira, J., Kinyua, Z., Olubayo, F., Karinga, J., Aura, J., Lees, A., Cowan, G., & Torrance, L. (2013). Occurrence and distribution of potato pests and diseases in Kenya. *Potato Research, 56*(4), 325–342. https://doi.org/10.1007/s11540-013-9246-9

Wratt, G., & Smith, H. (1983). *Plant breeding in New Zealand.* Wellington: Butterworth-Heinemann.

APPENDICES

Appendix I Map showing the agro-ecological zones in Kenya (courtesy: Kenya Soil survey)



Appendix II Definition of the agro-ecological zones described in this study. Table is adapted from agro-climatic zones of Kenya (1980)

Zone	**Mean annual temperature (°C)**	**Classification**	**Mean maximum temperature (°C)**	**Mean minimum temperature (°C)**	**Absolute minimum temperature (°C)**	**Night frost**	**Altitude (metres)**
Midlands (ML)	18 to 24	Warm temper-ate, fairly warm and warm	24 to 30	12 to18	4 to 10	none	1500 -1850
Lower Highlands (LH)	14 to 18	Fairly cool, cool temperate	20 to 24	8 to 12	0 to 4	Rare, very rare	1850- 2450
Upper Highlands (UH)	10 to 14	cool temperate	16 to 20	4 to 8	0 to - 4	Common, occasional	2450 - 3050

Appendix III Questionnaire for farmers

Details of interviewee

Name……**ID**…….

Gender……**Age**……

County……**GPS**……..

Contact details……**Date**…….....

A. Details of the farm

1. Host information: cultivar; age of trees; propagation method; land preparation; dates and rates of fertilizer application; pesticide history and schedule; pruning; transplanting; omissions or additions to conventional culture program?

2. How many acres of tree tomato have you planted and approximately how many trees do have you planted?

3. On average, how many kg do you harvest in a year?

4. What is the current selling price of tree tomatoes in the market?

5. What production constraints have you encountered so far in tree tomato farming?

6. Where do you sell your produce, local market or do you export?

B. Diseases

7. What diseases have constrained your production so far?

8. What symptoms have manifested so far?

9. At what age of the plant do the symptoms manifest?

10. What percentage of trees in the orchard manifest such symptoms?

11. Date of first symptoms and rate of syndrome development, coincident with any treatment or environmental event.

12. What methods have you used to try and control the disease and have they been effective?