Addis Ababa University
School of Graduate Studies
Institute of Biotechnology

# Distribution and Genetic Diversity of Maize (*Zea mays* L.) Associated DNA and RNA viruses in Ethiopia

**PhD Dissertation**

**Demsachew Guadie Tseganeh**

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# Distribution and Genetic Diversity of Maize (*Zea mays* L.) Associated DNA and RNA Viruses in Ethiopia

Demsachew Guadie Tseganeh

A Thesis Submitted to the Institute of Biotechnology, School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

Supervisors: Adane Abraham (PhD, Assoc. Prof.)
KassahunTesfaye (PhD, Assoc. Prof.)

# ADDIS ABABA UNIVERSITY
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**By**

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Approved by Examining Board:

| **Name** | **Signature** | **Date** |
|---|---|---|
| 1. Dr. Adane Abraham (Advisor) | __________________ | _______________ |
| 2. Dr. Kassahun Tesfaye (Advisor) | __________________ | _______________ |
| 3. Prof. Hassan K. Were (External Examiner) | __________________ | _______________ |
| 4. Dr. Tileye Feyissa (Internal Examiner) | __________________ | _______________ |
| 5. Dr. Tesfaye Sisay (Chairman) | __________________ | _______________ |

# Declaration

I, Demsachew Guadie Tseganeh, hereby declare that this dissertation and its entirety is my bona fide work and has been submitted in partial fulfillment of the requirements for a PhD degree at Addis Ababa University. This dissertation or its part has not been submitted to other University anywhere for the award of any degree. All sources of materials used for the dissertation have been duly acknowledged.

10 October, 2019

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## Abstract

Maize (*Zea mays* L.) is leading cereal crop in terms of production in Ethiopia. However, achieving its yield potential is hindered by a number of biotic and abiotic factors. To enhance its productivity, these limiting factors should be addressed. Among the biotic factors, more than 50 viruses can infect and cause disease on maize. Of these, the rapidly emerged maize lethal necrotic disease (MLND) and maize streak disease (MSD) were reported to have high yield loss potential. This study was initiated to survey maize fields, study associated virus diseases and examine their diversity in Ethiopia. A total of 284 maize fields from five major maize growing regions were surveyed in four survey missions between 2015 and 2017, of which 846 leaf samples with virus like disease symptoms were collected from 191 fields. Ninety three fields with no observable symptoms were exempted from sampling. Up to 100% disease incidence was recorded in the Benishangul-Gumuz, Oromia and South Nations, Nationalities and People (SNNP) regions. In the first set of test experiment double or triple antibody sandwich enzyme-linked immuno-sorbent assay to test for eight common maize viruses confirmed the presence of *Maize chlorotic mottle virus* (MCMV), *Sugar cane mosaic virus* (SCMV) and *Maize streak virus* (MSV). MLND was the most important disease in SNNP and Oromia while MSD was predominant in Benishangul-Gumuz. Sequence analysis of coat protein genes of these three viruses showed little variability. In the test experiment for mastreviruses, three genetic groups, each representing distinct virus species were identified. The first group represented the A-strain of MSV. The second sequence group shared 96-98% identity with *Maize streak reunion virus* (MSRV) isolates, confirming the presence of MSRV also in continental East Africa for the first time. Sequence analysis of additional virus genomes (each 2846 nt) representing the third group revealed only a limited nucleotide identity of 70-71% with MSRV isolates belonging to a novel virus species tentatively named maize streak dwarfing virus (MSDV). PCR screening of 89 samples showing streak symptoms with designed general or specific mastrevirus primers showed that MSV is the most incident followed by MSRV and MSDV. *Maize yellow mosaic virus* (MaYMV) was also assessed from 47 leaf samples by RT-PCR using general polerovirus primer pairs embracing 325 nucleotides of the coat protein gene 34 samples were positive for this primer. Direct sequencing of the RT-PCR products confirmed ≥ 99% nt identity to each other and shared 98 to 99% identity with the reference sequence, KU248489. Full-genome sequence variability study of three MaYMV isolates using Illumina MiSeq sequencing revealed 99.6% nt identity to each other. One of the complete genome sequences, MF684369 shared 96.8% nt identity with KU248489. In conclusion, this study revealed the presence of six viruses in maize fields of Ethiopia, their distribution, incidence and provided sequence data for these viruses. Moreover, occurence of three different mastrevirus species on maize in Ethiopia, is unprecedented, and suggests that Ethiopia may be one of the potential hot spots for the diversity of maize mastreviruses.

**Key Words/Phrases**: ELISA, Illumina MiSeq, Incidence, Maize viruses, RCA

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All happened with the Will of God!

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## List of Acronyms

| | |
|---|---|
| ABTS | 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ABTS 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) |
| AP | Alkaline phosphatase |
| CP | Coat protein |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| DAS-ELISA | Double Antibody Sandwiched Enzyme Linked Immunosorbent Assay |
| DBIBA | Dot-Blot Immuno Binding Assay |
| DEP | Dilution End Point |
| DSMZ | Deutsche Sammlung von Mikroorganismen und Zellkulturen (German collection of microrganisms and cell culture) |
| ELISA | Enzyme linked immunosorbent assay |
| ESA | Eastern and Southern Africa |
| HDA | Helicase dependent amplification |
| HRP | Horseradish peroxidase |
| ICTV | International Committee on Taxonomy of Viruses |
| ISEM | Immunosorbent Electron microscopy |
| IgGs | Immunoglobulin G |
| LFAs | Lateral flow assays |
| LIV | Longevity *in vitro* |
| LAMP | Loop-mediated isothermal amplification |
| MEGA | Molecular Evolutionary Genetic Analysis |

| | |
|---|---|
| NASBA | Nucleic acid sequence based amplification |
| NGS | Next generation sequencing |
| OPD | o-phenylenediamine dihydrochloride |
| pNPP | para-nitrophenyl phosphate, disodium salt |
| QCMI | Quartz crystal microbalance immunosensors |
| RAM-AP | Rabbit Ig anti-mouse Ig alkaline phosphatase |
| RCA | Rolling Circle Amplification |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| RPA | Recombinase Polymerase Amplification |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM | Scanning electron microscope |
| SNNP | Southern Nations, Nationalities and People |
| SSA | Sub-Saharan Africa |
| TAS-ELISA | Triple Antibody Sandwich |
| TBIA | Tissue-Blot Immuno Assay |
| TEM | The electron microscope |
| TIP | Thermal Inactivation Point |
| TMB | 3,3',5,5'-Tetramethylbenzidine |
| UNDP | United Nations Development Programme |

# Chapter 1

# 1. Introduction

## 1.1 General Introduction

To transform the world by 2030, the United Nations has set 17 sustainable development goals (SDGs) in 2015. Of these, the second goal is planned to ‘end hunger, achieve food security and improved nutrition and promote sustainable agriculture’ through doubling agricultural food productivity and ensuring sustainable food supply (UNDP, 2015). To achieve this goal, proven technologies which enhance food production and productivity should be employed and limiting factors should be addressed and resolved.

Maize (syn. corn) (*Zea mays* L.) is the third most important mega cereal crop cultivated worldwide next to wheat and rice (Dowswell *et al*., 1996). It is used for three main purposes: on one hand, in the developing world, it is used as a staple food crop for human consumption on the other hand as a feed for livestock and as raw material for many industrial uses, including bio-fuel production in the developed countries (Nafziger, 2010; Bekele Shiferaw *et al*., 2011). Maize is leading cereal in terms of production; produced globally across temperate and tropical zones spanning all continents. It provides at least 30% of the food calories of more than 4.5 billion people in 94 developing countries. Maize alone contributes over 20% of total calories in human diets in 21 low-income countries, and over 30% in 12 countries that are home to a total of more than 310 million people. Of the 22 countries in the world where maize forms the highest percentage of calorie intake in the national diet, 16 of them are in Africa (Nuss and Tanumihardjo, 2011). Therefore, it is vital crop for food security in Africa.

Maize, teff (*Eragrostis tef*), sorghum, wheat, and barley among cereals and enset (*Ensete ventricosum*) ("false banana") among "roots and tubers" provide the main calorie requirements in the Ethiopian diet (Tsedeke Abate *et al*., 2015).

Different biotic and abiotic constraints are known to negatively impact the yield of maize per unit area. Some of the common biotic factors include insect pests such as the stem borer *Chilo partellus* (Swinhoe) (Naz *et al*., 2003; Gupta *et al*., 2010), different species of weeds, and emerging diseases caused by fungi, bacteria, viruses and other agents. The common fungal diseases of maize are mainly classified as blights, blotches, ergotism, rots, rusts, smut and spots. Diseases caused by bacteria include stalk or seed rot, leaf spot, stewat's wilt, leaf blight and bacterial leaf stripe (De Leon, 1984).

Reportedly, more than 50 species of virus have been identified to infect and cause disease on maize (Redinbaugh and Zambrano, 2014). The diseases caused by these viruses are estimated to cause average 3% annual yield losses which is equivalent to $8 billion USD in 2012 (Oerke and Dehne, 2004). Since the occurrence of maize virus diseases is spotty, in some cases, losses can be much higher in certain geographical locations. For example, in 2011, smallholder farmers in the Longisa division of Bomet County, Southern Rift Valley, Kenya experienced more than 80% losses due to maize lethal necrosis disease (MLND), a disease caused by co-infection of maize with *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV) (Wangai *et al*., 2012a).

There are different maize virus diseases reported from Ethiopia (Mesfin Tessera *et al*., 1991; Alemu Lencho *et al*., 1997; Mahuku *et al*., 2015b; Mengistu Fantahun *et al*., 2017). The devastating nature of currently emerged virus disease of maize, MLND, since its first report in Africa from Kenya in 2011 (Wangai *et al*., 2012a) and rapid expansion to the Eastern region of Africa including Ethiopia alerted us to explicitly survey and characterize the DNA and RNA viruses associated with maize in Ethiopia. Therefore, this thesis was conducted to consecutively survey viruses associated with maize in major maize growing regions of Ethiopia in a comprehensive approach, identify their distribution using serological and molecular techniques, and characterize them by providing information on partial as well as full-genome sequences of common or novel maize viruses from the same country.

## 1.2 Objectives of the study

- The overall objective of this study was to determine the distribution of DNA and RNA viruses of maize among major maize growing regions of Ethiopia and to characterize these viruses using serological and/or molecular approaches.

### 1.2.1 Specific Objectives

- ✓ To describe maize viruses from major maize growing regions of Ethiopia (Amhara, Benishangul-Gumuz, Oromia, Southern Nations, Nationalities and People (SNNP) and Tigray) and study their infections;
- ✓ To identify MLND causing and other common maize viruses and study their geographical distribution and coat protein gene variability in Ethiopia;
- ✓ To characterize maize (*Zea mays* L.) infecting Mastreviruses;
- ✓ To study *Maize yellow mosaic virus* in Ethiopia using molecular techniques.

# Chapter 2

# 2. Literature Review

## 2.1 Origin and Distribution of Maize

Maize, also called corn, is one of the oldest domesticated plant species and thought to originate in the Mesoamerican region, Mexico and Central America (Watson and Dallwitz, 1992). The term corn assumed to originate from the Germanic term 'korn' meaning any edible grass (Abbassian, 2007). There have been several principal and minor theories regarding the origin of maize. Among these, (1) tripartite hypothesis, (2) catastrophic sexual transmutation theory, (3) *Tripsacum-Zea diploperennis* hypothesis and, (4) teosinte hypothesis are commonly described. Species of the two genera, *Teosintes* (*Z. diploperennis* and *Z. mays* ssp. *mexicana*) and *Tripsacum* are usually termed as having high contribution for the origin of maize. An early hypothesis proposed that *Z. mays* ssp. *mexicana* was the result of a natural hybridization of *Tripsacum* and *Zea* (Mangelsdorf, 1974). Further crossings of *Teosinte* with wild maize are thought to have produced the modern races of maize. The possibility of intergeneric hybridization of either *Z. diploperennis* or *Tripsacum* with missing link extinct wild maize has also been proposed as the ancestral origin of *Z. mays* (Purseglove, 1972; Radu *et al.*, 1997).

Eubanks (1997) also suggested that domesticated maize may have arisen via human selection of natural hybrids between *Teosinte* and *Tripsacum*. As revealed from archaeological records and phylogenetic analysis, domestication of maize began at least 6000 years ago, arising independently in regions of the Central America, Mexico and southwestern United States (Piperno and Flannery, 2001; Matsuoka *et al.*, 2002).

Maize was introduced to the temperate region from Central and Southern America in the 15th century following the return of Columbus from America. It spread out from the Europe to Africa and Far East Asia fundamentally through trade and colonialism (Farnham *et al*., 2003). It was introduced to the African continent through West Africa by Portuguese traders in the 1500s (Dowswell *et al*., 1996) and brought to Ethiopia in the 1600s to 1700s (Haffangel, 1961). Its adaptability to varied environmental conditions is far better than other crops. It grows from 58° N to 40° S latitude and in altitude ranges from sea level to 3000 m.a.s.l. and in areas with rainfall range of 250 mm to 5000 mm per year (Dowswell *et al.*, 1996).

## 2.2 Morphology and Reproductive Biology of Maize

Maize is an annual monocot plant varying in height from one to four meters with large, narrow, opposite leaves growing alternately along the nodes of erect stem (culm). The ear bearing inter node is longitudinally grooved to allow proper positioning of the ear head (cob). It has three root systems. The first one is seminal root which develops from radicle and persists for long time; the next is adventitious roots which are active and operative fibrous roots of the plant growing from the lower nods of stem below ground level. The third groups are the brace or prop roots which are produced by lower nodes (Tripathi *et al.*, 2011).

Maize is a monoecious plant bearing pistillate (ear) and staminate (tassel) flowers partitioned on the same plant in which the main shoot terminates in a tassel. Maize flower is protandrous type, that is, the male flower matures earlier than the female one. Within each male flower spikelet, there are usually two functional florets, although the upper floret develops earlier than the lower floret. Each floret contains a pair of thin scales called lemma and palea, three anthers, two lodicules and rudimentary pistil. Pollen grains per anther have been reported to range from 2000 to 7500 (Dhillon and Prasanna, 2001).

The female inflorescence develops from one or more lateral branches (shanks) usually borne about half-way up the main stalk from auxillary shoot buds. The female flower which is initially smooth but protuberances soon form in rows. The basal protuberances are formed first and development advances towards the tip of the ears. The part above the attachment of the carpel develops a single sessile ovule, which consists of a nucellus with two integuments. The united carpels, which will form the ovary wall or pericarp of the mature kernel, grow upward until they completely enclose the ovule. The two anterior carpels, which face the ear tip, form outgrowths, which develop into the style known as silks. Silks are covered with numerous hairs, trichomes which form an angle with the silk where pollen grains are harbored. The base of the silk is unique, as it elongates continuously until fertilization occurs. The cobs bear many rows of ovules that are always even in number (Kiesselbach, 1949).

The presence of specialized anatomical and biochemical features improved the photosynthetic efficiency of maize. This trait is shared by few other crops, including sorghum and sugarcane which are collectively called C4 plants (Nafziger, 2010).

There are several hybrids of maize, each with specific properties and kernel characteristics. The most common ones include: dent (or field maize, used for livestock feeding and can be yellow or white), flint (or Indian maize, grown mostly in Central and South America), and sweet (or green maize). Based on colour and taste, maize grown around the world is generally grouped into two: yellow and white. Yellow maize constitutes the majority of total world production and international trade. It is grown in northern hemisphere countries where traditionally used as animal feed. White maize is produced in some countries including the United States, Mexico and in southern Africa and generally considered as a food crop (Abbassian, 2007).

## 2.3 Taxonomy and Genetics of Maize

Maize (*Zea mays* L.) belongs to the genus *Zea* in the family *Poaceae* (*Gramineae*). The genus *Zea* consists of five species including *Z. diploperennis*, *Z. perennis*, *Z. luxurians*, *Z. nicaraguensis*, and *Z. mays*. The species *Zea mays* is divided into four sub-species: *huehuetenangensis*, *mexicana*, *parviglumis* and *mays* of which the sub-species *mays* is economically important and the rest three sub-species are collectively called teosintes. Teosintes are assumed progenitor of maize which are largely wild grasses native to Mexico and Central America (Mangelsdorf *et al*., 1981; Doeblay, 1990).

Maize is an outcrossing species, which makes its genetic architecture such as diversity, linkage, recombination, etc. more similar to other outcrossing organisms such as humans rather than self-pollinating plants (Rafalski and Morgante 2004; Wallace *et al*., 2013).

Except for *Z. perennis* (a perennial teosinte with 2n = 40), species in the genus *Zea* have a diploid number of 20 chromosomes (2n = 20) (Tito *et al*., 1991; Molina and Gracia, 1999; Ellneskog-Staam *et al*., 2007; Khalili *et al*., 2013). Maize has large genome which is rich in transposons and its size ranges from 2.3 - 2.7 Gbp with a total gene number of 42,000 - 56,000 (Doeblay, 1990; Arumuganathan and Earl, 1991).

## 2.4 Production of Maize

Maize is used as food, feed and raw material in food manufacturing industries. Some of the industrial products produced from maize include starch, oil, syrup, alcohol, acetic and lactic acids, glucose, paper, rayon, plastic, textile, adhesive, dyes, synthetic materials, rubber and maltodextrins. It is used as biofuel (Tripathi *et al*., 2011). Moreover, maize serves as a model organism for biological research worldwide. Mexico (1994) indicated that maize is used for different purposes in different countries. In the developed countries it is mainly used as animal feed or raw material in food manufacture industries. In most of the developing countries however, it is mainly used for food though it is used both for food and feed in Asia.

Maize grows in diverse agro-ecological zones and farming systems, and consumed by people with varying socio-economic backgrounds and food preferences in sub-Saharan Africa (SSA). It plays a vital role as a staple food in SSA which can be comparable to that of rice or wheat in most parts of Asia, with consumption rates being the highest in eastern and southern Africa (ESA). Maize accounts for almost half of the calories and protein consumed in ESA. An estimated number of 208 million people in SSA depend on maize as a source of food security and other sources of economy. Out of the SSA's estimated 200 million ha of cultivated land, maize occupies more than 33 million ha (Bekele Shiferaw *et al*., 2011).

### 2.4.1 Global Production

Maize is the third most important agricultural commodity worldwide. Different varieties are used for human consumption, animal feed, and as raw materials for industrial processing and manufacturing. Originally planted in Middle America, due to its geographical adaptability and climatic resistance, maize has successfully spread all around the globe with particular relevance in Latin America and SSA (Abbassian, 2007). However, its premier productivity surpasses all other cereal crops (FAOSTAT, 2017).

It is the main staple food for more than 1.2 billion people in Sub-Saharan Africa and Latin America (Iken and Amusa, 2004). Maize contains about 72% starch, 10% protein, and 4% fat, providing an energy density of 365 Kcal/100 g, as compared to rice and wheat, but it has lower protein content (Nuss and Tanumihardjo, 2010). Maize is rich in many of the vitamin B-complexes and essential minerals along with fiber, but it lacks some other nutrients, such as vitamin B12 and vitamin C and is, in general, a poor source of calcium, folate, and iron (Ranum *et al*., 2014).

Maize is grown over a wider range of altitudes and latitudes than any other food crop, under temperatures ranging from cool to very hot, on wet to semi-arid lands, and in many different types of soils. Maize is an important source of food and nutritional security for millions of people in the developing world, especially in Africa and Latin America. It accounts for nearly half of the calories and protein consumed in Eastern and Southern Africa (ESA) and one-fifth of calories and protein consumed in West Africa (Bekele Shiferaw *et al*., 2011). Except in the years 2009 and 2012, the global production of maize showed progressive increament in the past 10 years back from 2017 (Table 2.1).

**Table 2.1** Global production status of maize from 2008 to 2017.

| Year | Production (Tonnes) | Area Harvested (Ha) | Yield (Hg/Ha) |
|---|---|---|---|
| 2017 | 1134746667 | 197185936 | 57547 |
| 2016 | 1100225518 | 195363162 | 56317 |
| 2015 | 1052097073 | 190435913 | 55247 |
| 2014 | 1039267776 | 185807919 | 55932 |
| 2013 | 1016207182 | 186957444 | 54355 |
| 2012 | 875039160 | 179791974 | 48670 |
| 2011 | 886680581 | 171202475 | 51791 |
| 2010 | 851679519 | 164020015 | 51925 |
| 2009 | 820072448 | 158819581 | 51635 |
| 2008 | 829240208 | 163142954 | 50829 |

Source: FAOSTAT, 2008-2017.

### 2.4.2 Maize Production in Ethiopia

Maize is Eth.iopia’s leading cereal crop in terms of production with 8.396 million tons produced in 2018 by 10.574 million farmers across 2.129 million hectares of land with an average yield of 3.944 tons per hectare (CSA, 2018). In terms of cultivated area, it is the second most widely cultivated crop next to tef [*Eragrostis tef* (Zucc.) Trotter]. Over half of all Ethiopian farmers grow maize, primarily for subsistence with 75% of all maize output consumed by farming households, making it a key crop for overall food security and for economic development in the country. It is cultivated in a wide range of altitudes, moisture regimes, soil types and terrains, mainly by smallholder crop producers. Over the last 20 years, the area under maize cultivation has increased by about 50% and production by 66%, with the national average yield of maize increasing from 1.6 to 3.9 t/ ha (CSA, 2018).

The wide adaptability of maize to wide agro-ecological zones and its potential to produce more calories and food per area of land cultivated than all major cereals grown in Ethiopia were important factors in considering maize as part of the national food security strategy. It is increasingly used both separately as well as in mixed flour with other more expensive cereals in traditional Ethiopian diets (Tsedeke Abate *et al*., 2015). Maize is the most important staple in terms of calorie intake in rural Ethiopia. The 2004/5 national survey of consumption expenditure indicated that maize accounted for 16.7 % of the national calorie intake followed by sorghum (14.1 %) and wheat (12.6 %) among the major cereals (Guush Berhane *et al*., 2011).

Because of its high value as a food crop as well as the rising demand for the stover as animal fodder and source of fuel for rural families, maize is a popular crop in Ethiopia. Nearly 88 % of maize produced in Ethiopia is consumed as food, both green and dry grain. Maize for industrial use has also growing demand and the rapidly growing urbanization as well as poultry industry is demanding maize grain. In recent years, Ethiopia has increasingly attained self-sufficiency in maize production and even exported to neighboring countries such as Sudan and Djibouti when there is surplus production (Tsedeke Abate *et al*., 2015). As described by Zeng *et al*. (2015) this increased productivity and production of maize has a significant contribution on poverty reduction. The production of maize in Ethiopia has successively increased from 2008 to 2017 (Table 2.2).

**Table 2.2** Maize production status in Ethiopia from 2008 to 2017.

| Year | Production (Tonnes) | Area Harvested (Ha) | Yield (Hg/Ha) |
|---|---|---|---|
| 2017 | 8116787 | 2173543 | 37344 |
| 2016 | 7847175 | 2135572 | 36745 |
| 2015 | 7882444 | 2111518 | 37331 |
| 2014 | 7234955 | 2114876 | 34210 |
| 2013 | 6491540 | 1994813 | 32542 |
| 2012 | 6158318 | 2013045 | 30592 |
| 2011 | 6069413 | 2054724 | 29539 |
| 2010 | 4986125 | 1963180 | 25398 |
| 2009 | 3897163 | 1772253 | 21990 |
| 2008 | 3776440 | 1767389 | 21367 |

Source: FAOSTAT, 2008-2017.

## 2.5 Maize Production Constraints

### 2.5.1 Abiotic Stresses

Abiotic stresses are integral part of any agro-ecosystem and affect crop plant in variety of ways. Most common abiotic stresses include soil type, temperature, relative humidity, organic matter in the soil and precipitation. Drought is the most important abiotic stress in maize production and productivity in the tropical and sub-tropical regions (Bekele Shiferaw *et al*., 2011).

Due to very low use of fertilizer and the demise of bush-fallow systems of soil fertility replenishment, soil fertility is also even more serious constraint in most maize growing environments in Africa (Morris *et al*., 2007). The interlinked soil fertility problems of acidity, aluminium toxicity, and low phosphorous availability constrain yields on about 4 million hectare of cropland worldwide. Excess rainfall and poor drainage is another severe constraint to maize productivity, particularly during the wet season in areas of Asia that are heavily dependent on monsoon rainfall. In such conditions, maize yields are often approximately one-half to one-third lower than irrigated production in the dry season. The combinations of drought, heat or cold stress and water-logging/excess moisture, coupled with vulnerability to emerging diseases and insect-pests, are likely to increase in some regions, especially in Sub-Saharan Africa and Asia, with global climate changes (Bekele Shiferaw *et al*., 2011).

### 2.5.2 Biotic Stresses

Biotic stress is one of the major environmental factors adversely affecting crop production and productivity. The major biotic stresses include diseases caused by fungi, bacteria, viruses, nematodes, or phytoplasmas, insect-pests and weeds (Gupta *et al*., 2010).

Maize diseases of global or regional importance include southern corn leaf blight (*Bipolaris maydis*), southern rust (*Puccinia polysora*), northern corn leaf blight (*Exserohilum turcicum*), common rust (*Puccinia sorghi*), gray leaf spot (*Cercospora species*), stalk and ear rots caused by *Diplodia* and *Fusarium*, and kernel and ear rots caused by several *Fusarium* and *Aspergillus* species, which also contaminate grain with mycotoxins thereby reducing grain safety and quality. Diseases that are particularly important in Asia are the downy mildews, post-flowering stalk rots (PFSR), gray leaf spot, banded leaf and sheath blight (BLSB), and turcicum leaf blight. For Latin America, the tar spot complex and the corn stunt complex diseases are of particular importance. Major biotic stresses limited to Africa include *maize streak virus* (MSV), and the parasitic weed Striga (*Striga asiatica* and *Striga hermonthica*) (Bekele Shiferaw *et al*., 2011).

The sudden out break and rapid distribution of the invasive alien species which were common in USA are also great challenges in the African maize production system. Practical examples of these alien species include MCMV (Wangai *et al*., 2012a) which result in the devastating disease, MLND up on its synergestic interaction with either of the potyviruses: WSMV, MDMV, JGMV or SCMV. The fall armyworm (FAW; *Spodoptera frugiperda* (JE Smith); *Lepidoptera*, *Noctuidae*) is also another alien invasive species. It was first reported in Nigeria and South Africa on the African continent in January 2016 (Goergen *et al*., 2016). In the next year, the insect moved all the way to East Africa and found in Ethiopia in March 2017; and is now confirmed in more than 30 countries of the continent (Prasanna *et al*., 2018).

## 2.6 Plant Viruses

Compared to other plant pathogens including bacteria and fungi, viruses are very tiny particles which cannot be seen without the aid of transmission electron microscope. They are obligate parasites and simply consist of an inner genetic material (either DNA or RNA) enclosed with an outer protein coat usually referred as capsid (Jeong *et al*., 2014). The capsid is made up of many protein subunits of one or more types called capsomeres and protect the nucleic acid from enzymatic degradation. Although a number of plant and animal viruses have been described to date, the first virus ever described since the late 1880s was *Tobacco mosaic virus* (TMV). The term virus was derived from the original description of the causal agent of TMV a "*contagium vivum fluidum*" meaning contagious living fluid. Eventually the term virus was coined and used from the Latin word 'venom' denoting poisonous fluid (Beijerinck, 1898).

According to the 9th report of International Committee on Taxonomy of Viruses (ICTV), there are about 2284 reported virus and viroid species distributed amongst 349 genera, 19 subfamilies, 87 families and 6 orders. Nearly half of these known viruses can attack and cause diseases in plants. One virus may infect one or more species of plants. A plant may sometimes be infected by more than one virus species at the same time (King *et al*., 2012).

Plant viruses are usually classified based on the type, composition and orientation of nucleic acid they contain. Virus genomes are composed of either RNA or DNA. They are further sub-divided depending on whether they possess one or two strands of RNA or DNA of either positive or negative sense, either filamentous or isometric. Most plant viruses have positive sense single stranded RNA (ssRNA) genome. Some however, contain double stranded DNA (dsDNA) with the same helical structure as in host plant cells. Others contain single stranded DNA (ssDNA) or double stranded RNA (dsRNA) which occurs either in linear strands or in circular forms. If the ssRNA of the virus contains a sequence of nucleotides that directly codes for a protein, it acts in a similar fashion to messenger RNA (mRNA) and the virus is said to have a positive sense genome. If it has a nucleotide sequence complementary to that of the virus mRNA it is said to have a negative sense genome. A few virus RNAs code for some proteins in the positive sense and others in the negative sense and are described as having ambisense genomes. Within each of these groups there may be viruses replicating via a polymerase enzyme (+RNA or DNA viruses) or via a reverse transcriptase (-RNA or DNA viruses) (Agrios, 2005). Plant viruses cause a significant proportion of crop diseases and economic losses around the world (Gomez *et al*., 2009).

### 2.6.1 Common Maize Viruses

More than 50 viruses have been identified to infect and cause diseases on maize (Lapierre and Signoret, 2004). Due to advances in virus diagnostic tools a lot of novel specieses have also been discovered on wards. Some of commonly occurring maize viruses are described below:

*Maize dwarf mosaic virus* (MDMV)

MDMV belongs to the genus *Potyvirus* in the *Potyviridae* family. The virus particle is flexuous rod-shaped, 750 nm in length and 12-15 nm in diameter. It was identified for the first time in 19 60's in Illinois (Tosic *et al*., 1990). MDMV has a positive ssRNA genome which is ~ 9.5 kb in size (Achon *et al*., 2012). A large polyprotein (338 kDa) is translated from a single open reading frame (ORF), which is later proteolytically cleaved by three self-coded proteinases to yield 10 final proteins (P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb, and CP) with multiple functions (Kong *et al*., 1998; Urcuqui-Inchima *et al*., 2001). It is non-persistently transmitted by over 20 different aphid species (Knoke and Louie, 1981).

Five strains of MDMV (strains A, C, D, E and F) are recognized based on symptomatology on inbred maize lines such as inbred line N20 and frequency of transmission by different aphid species (Shukla *et al*., 1994). In general, MDMV can result in maize yield loss of up to 70% mainly due to a reduction in the rate of photosynthesis and elevation in the rate of respiration (Gates *et al*., 1969; Mikel *et al*., 1981). Among all the maize-infecting viruses, MDMV is the most common disease agent globally reported in Africa, United States, Asia and Europe (Gordon *et al*., 1981; Gell *et al*., 2010).

### *Sugarcane mosaic virus* (SCMV)

*Sugarcane mosaic virus* (formerly called MDMV-B) belongs to the largest genus in the *Potyviridae* family known as *Potyvirus*. It was first described by Brandes (1919) from sugarcane. Its single infection on maize causes maize dwarf mosaic disease (MDMD) (Janson and Ellett, 1963). It has flexuous filamentous particles of 700-750 nm long encapsidating positive sense, monopartite ssRNA encoding a single large polyprotein. It is non-persistently transmitted by aphids in non-circulative manner (King *et al*., 2012). Natural infection of SCMV is restricted to sugarcane, maize, sorghum and other species members of the *Poaceae* family.

### *Wheat streak mosaic virus* (WSMV)

WSMV belongs to the genus *Tritimovirus* in the *Potyviridae* family. It is both seed-borne and seed-transmitted, also vectored by the wheat curl mite *Aceria tosichella* Keifer (Acari: Eriophyidae). The mites overwinter on winter wheat then leave the crop as it matures and are blown by the wind to spring wheat, barley and corn (Nault *et al*., 1967). WSMV has also been found co-infecting maize together with MDMV (Hill *et al*., 1974). WSMV has a relatively broad host range, encompassing many plants in the grass family. Wheat is the preferred host for wheat curl mite and WSMV. Both mites and WSMV are found on other crops such as corn (*Zea mays* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), oat (*Avena sativa* L.), pearl millet (*Pennisetum glaucum* L.), and sorghum (*Sorghum bicolor* L.) (Seifers *et al*., 1995). WSMV and wheat curl mite also use a number of wild grass species as their host, but no dicotyledons have been found to host WSMV (Hadi *et al*., 2011).

### *Johnson grass mosaic potyvirus* (JGMV)

JGMV is a *Potyvirus* which is first reported in Johnson grass and maize from Australia and spreads in Australia and the USA. Natural hosts of JGMV include Johnson grass, maize and a number of grass species (Brunt *et al*., 1996). It is transmitted in a non-persistent manner by *Aphis craccivora*, *A. gossypii* and *Rhopalosiphum maidis*. It is not transmitted through seed (Brunt *et al*., 1996).

It has vast world wide distribution and reported from Africa in Nigeria (Seifers *et al*., 2005) as well as form Kenya as it is also contributing to maize lethal necrotic disease in the region (Stewart *et al*., 2017) and in Ethiopia (Alemu Lencho *et al*., 1997).

*Maize streak Virus* (MSV)

*Maize streak virus* (MSV), initially named by Storey in 1924, belongs to the genus *Mastrevirus*; family *Geminiviridae*. It is the causal agent of maize steak disease (MSD) which is important and wide spread disease affecting maize in SSA and different parts of the world in the Eastern Hemisphere (Bosque-Perez 2000; Magenya *et al*., 2008). It has a monopartite genome with four open reading frames approximately 2.7 kb in size and encapsidated in geminate particles (King *et al*., 2012).

It is persistently transmitted by nine leafhopper species in the genus *Cicadulina* of which *C. mbila* Naudé and *C. storey* are most common (Okoth *et al*., 1987; Asanzi *et al*., 1995). Among the 11 strains described, MSV A to K (Varsani *et al*., 2008), MSV A is usually infect and cause economically significant yield loss in maize (Willment *et al*., 2001; Owor *et al*., 2007; Shepherd *et al*., 2010). MSV A has five strain variants namely: MSV-A1, MSV-A2, MSV-A3, MSV-A4 and MSV-A6 and the rest strains (MSV B to K) are generally found infecting wild grass species (Varsani *et al*., 2008). MSV is indigenous to Africa, including the adjacent Indian Ocean Islands of Reunion, Mauritius and Madagascar (Willment *et al*., 2001; Fajemisin, 2003).

Apart from maize, MSV infects different species of crops such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), oats (*Avena sativa* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), finger millet (*Eleusine coracana* L.), pearl millet (*Pennisetum typhoides* L.), sorghum (*Sorghum bicolor* L.) and sugarcane (*Saccharum officinarum* L.) (Damsgeegt, 1983; Willment *et al*., 2001; van Antwerpen *et al*., 2011).

MSV replicates in the nuclei of infected cells by a method called rolling-circle replication (RCR) (Saunders *et al*., 1991) and through recombination-dependent mechanisms (Jeske *et al*., 2001). RCR is initiated by binding of the virus replication-associated protein (Rep) to the virion-strand origin of replication, where the protein initiates and terminates virion-strand DNA synthesis. Rep is the translation product of two complementary-sense open reading frames (ORFs), C1 and C2. The C1-C2 transcript, which contains an intron, is translated to yield either Rep (from the spliced transcript) or RepA (from the unspliced transcript). These proteins share several distinct domains with diverse biochemical activities, such as sequence-specific DNA binding, virion-sense origin cleavage and ligation (Heyraud-Nitschke *et al*., 1995) and potential transactivation of viral promoters (Collin *et al*., 1996). As Rep is the only viral protein essential for virus replication and is required early in the infection process, it is an ideal target for genetic engineering of MSV resistance in maize (Shepherd *et al*., 2007).

### *Maize chlorotic mottle virus* (MCMV) *and Maize lethal necrosis disease*

*Maize chlorotic mottle virus* (MCMV) is the sole type species of the genus *Machlomovirus* in the family *Tombusviridae*. It has single molecule of infectious, linear, positive sense and ssRNA genome which is about 4.4 kb, lacking 5’ cap or 3’ poly (A) tract. The genomic RNA contains four ORFs; ORF1, ORF2, ORF3 and ORF4 coding 32 kDa, 48 kDa, 7 kDa and 25 kDa proteins, respectively. Unlike other genus in the family, genome of MCMV encode an additional 5’-proximally located ORF encoding a 32 kDa protein of unknown function. MCMV lacks protein envelope and have spherical morphology with icosahedral symmetry. The virions are approximately 30 nm in diameter. Its host range is restricted to monocotyledons (King *et al*., 2012).

Single infection of MCMV, as a maize viral disease, was first identified in Peru in 1973 (Castillo and Hebert, 1974). Later its single infection has been reported in different parts of the world including: Argentina (Teyssandier *et al*., 1981), Thailand (Klingkong and Sutaburta, 1982), Mexico (Carrera-Martinez *et al*., 1989), Colombia (Morales *et al*., 1999) and China (Xie *et al*., 2011).

However, it’s pronounced disease severity, when it synergistically infects maize in association with any of the potyvirus MDMV, SCMV, WSMV and JGMV (Niblett and Clafin, 1978; Uyemoto *et al*., 1980; Stewart *et al*., 2017) has been reported and termed as corn lethal necrosis (CLN) or maize lethal necrotic disease (MLND). Corn lethal necrosis was first described in the United States where MCMV co-infect maize with MDMV primarily in north central Kansas and south central Nebraska (Niblett and Clafin, 1978; Uyemoto *et al*., 1980; Uyemoto, 1983) and later in Hawaii (Jensen *et al.*, 1990; Jiang *et al*., 1992). It is also reported from Taiwan (Deng *et al*., 2014) and Ecuador (Quito-Avila *et al*., 2016). Recent reports indicated the association of SCMV with MCMV to result in

MLND in China (Wang *et al*., 2017). MLND has also been reported from the warmer temperate zone in Spain as a result of synergistic interaction of MCMV with MDMV (Achon *et al*., 2017).

In Africa, MLND is first reported in 2011 from the Longisa Division of Bomet District, Kenya, as it appears co-infecting maize with MCMV and SCMV (Wangai *et al*., 2012a). It is alarmingly emerging to other countries of Africa including Tanzania (Wangai *et al*., 2012b), South Sudan (FAO, 2013), Democratic Republic of Congo (Lukanda *et al*., 2014), Rwanda (Adams *et al*., 2014) and Ethiopia (Mahuku *et al*., 2015b; Mengistu Fantahun *et al*., 2017; Demsachew Guadie *et al*., 2019). MLND has now emerged and spread as a menace to maize based food security in SSA (Mahuku *et al*., 2015a, Kiruwa *et al*., 2016). MLND has damaging effects on food security; especially for smallholder farmers and it can cause up to 100% yield loss in severe cases (Redinbaugh and Stewart, 2018).

Use of advanced techniques such as next-generation sequencing (NGS) for samples collected from MLND-affected areas from different East African countries have led to the discovery of additional, previously unknown viruses in maize. These viruses, which co-occur with MCMV, include *Amplelovirus*, *Closterovirus*, *Foveavirus*, *Partitivirus*, *Polerovirus*, *Pteridovirus*, *Totivirus* and others (Adams *et al*., 2017). However, symptomatology and synergistic interaction of these viruses with MCMV needs further description.

MCMV can experimentally be vectored in a semi-persistent way by several insect vectors including maize thrips (*Frankliniella williamsi*) (Jiang *et al*., 1992), maize root worms (*Diabrotica undecimpunctata, D. longicornis* and *D. virgifera*), cereal leaf beetles (*Oulema melanopus*), corn flea beetle (*Systena frontalis*) and *Chaetocnema pulicaria* (Nault *et al*., 1978; Cabanas *et al*., 2013). Jensen *et al*. (1991) reported a seed transmission rate of 0.33% for MCMV using maize seeds collected from MCMV infected plants. RT-PCR analysis of maize seeds collected from MCMV infected plants indicated 18 of 25 seeds (72%) positive seeds in Kenya but this may not directly correlate with the transmission potential to the progenitors (Mahuku *et al*., 2015a).

### *Maize mosaic virus* (MMV)

Maize mosaic virus (MMV; family *Rhabdoviridae*; genus *Nucleorhabdovirus*) was first reported from Venezuela in 1960 (Herold *et al*., 1960) and commonly found in tropical and sub-tropical regions of Africa, the Americas, and Hawaii thereafter. It is persistently transmitted by the primary vector, the maize plant hopper (*Peregrinus maidis* Ashmead) (Brewbaker, 1979). MMV has a unipartite, negative sense ssRNA genome and bullet-shaped, membrane-bound virions (Redinbaugh *et al*., 2012).

No seed transmition is reported for MMV (Falk and Tsai, 1985). Maize and teosinte are the major reservoir hosts of MMV, moreover, itchgrass (*Rottboellia cochinchinensis*), and plains bristlegrass (*Setaria vulpiseta*) are natural hosts for this virus (Redinbaugh and Zambrano, 2014).

### *Maize rayado fino virus* (MRFV)

*Maize rayado fino virus* is type member of the genus *Marafivirus*, family *Tymoviridae.* It was identified in the late 1960s in El Salvador and Costa Rica and causes maize rayado fino disease (Gamez, 1969). The virus has a monopartite ssRNA genome with a 30-nm icosahedral virion (Lapierre and Signoret, 2004). MRFV is naturally transmitted in a persistent propagative manner by the maize leafhopper, *Dalbulus maidis* (Rivera and Gamez, 1986). Maize rayado fino symptoms include small chlorotic spots on young leaves that become elongated and more numerous along leaf veins as the plant grows and result in stunted growth and grain yield reduction (Bustamante *et al*., 1998; Vasquez and Mora, 2007).

### *Maize stripe virus* (MSpV)

*Maize stripe virus* (MSpV; genus *Tenuivirus* in the genus *Tenuiviridae*) which causes maize stripe disease was discovered in Florida in 1970's (Tsai, 1975) and found distributed in many tropical areas of the world after wards (Ammar *et al*., 1995; Falk and Tsai, 1998). It has non-enveloped five segmented ssRNA genome RNA-1 (about 9 kb, generally of negative polarity), RNA-2 (3.3-3.6 kb, ambisense), RNA-3 (2.2-2.5 kb, ambisense), RNA-4 (1.9-2.2 kb, ambisense) and RNA-5 (1.3 kb, negative polarity) (Falk and Tsai, 1998; King *et al*., 2012).

This virus is persistently transmitted by the delphacid plant-hopper, *Peregrinus maidis*. Natural hosts of MSpV include maize, sorghum, and itch grass (*Rottboellia exaltata*). Symptoms as a result of stripe disease are fine chlorotic stipplings between leaf veins which later develop into continuous chlorotic stripes of varying width and intensity (Greber, 1981).

### *Maize rough dwarf virus* (MRDV)

*Maize rough dwarf virus* (MRDV) belongs to genus *Fijivirus* in the family *Reoviridae*. MRDV together with the related species *Rice black streaked dwarf virus* (RBSDV) causes maize rough dwarf disease (Lapierre and Signoret, 2004). The genomes of these viruses consist of 10 dsRNA segments that are encased in complex and spherical virions (Fauquet, 2005). The total nucleotide sequence of these 10 segments was found to be about 29 kb (Zhang *et al*., 2001; Lv *et al*., 2016).

Both MRDV and RBSDV are transmitted by the small brown planthopper (*Laodelphax striatellus* Fallen) in a persistent and propagative manner. Symptoms as a result of infection with MRDV include irregular growths (enations) on the underside and sheaths of leaves and ears that make them rough, dark green colored leaves, poorly developed root systems and shortened internodes leading to dwarfing, and they are far less productive compared to healthy maize (Lv *et al*., 2016). Both MRDV and RBSDV infect oats, wheat, crabgrass, bermudagrass (*Cyndon dactylon*), and cockspur (*Echinochloa crus-galli*) (Redinbaugh and Zambrano, 2014). MRDV naturally infects maize and causes a severe and economically important disease mainly in Europe and the Middle East (Dovas *et al*., 2004).

*Maize chlorotic dwarf virus* (MCDV)

Maize chlorotic dwarf virus (MCDV; family *Secoviridae*, genus *Waikavirus*) is among the virus diseases of maize that emerged rapidly and destructively in the 1960s in USA causing stunting symptoms (Rosencranz, 1969; Redinbaugh and Zambrano, 2014). MCDV has a positive sense ssRNA genome with about 11.8 kb in size. It is a phloem-limited virus primarily transmitted in a semi-persistent manner by black-faced leafhopper *Graminella nigrifrons* (Forbes) and no recorded evidence for seed transmission (Nault *et al*., 1973).

Besides maize, the primary natural and reservoir hosts of MCDV are Johnsongrass, sorghum, wheat, *Setaria* spp., and crabgrass (*Digitaria saguinalis* L.). Characteristic symptoms of MCDV include plant stunting and dwarfing, vein banding in leaves, leaf twist and tear, chlorosis and reddening or yellowing (Redinbaugh and Zambrano, 2014).

## 2.7 Plant Virus Detection Methods

Plant viruses cause major crop yield losses around the globe. It is not possible to control plant viral diseases using chemical agents. Strategies for virus disease management are mostly aimed at eradicating the source of infection or using virus free propagules, rouging of infected individual plants, elimination of alternative hosts, interfering with the movement of vectors to prevent the spread of disease and by quarantine practices. However, the most effective means of controlling virus diseases is through cultivation of resistant varieties. Precise identification of the causal agent is the first step in management of plant diseases (Webster *et al*., 2004; Adams *et al*., 2013).

The diagnosis step is the basis to manage plant diseases and to predict the crop loss as a result of infection (van der Want and Dijkstra, 2006). Accurate diagnosis of virus diseases is the first important step for crop management system. In order to prevent plant viral diseases, it is important to figure out the causative agents and distinguish diseased plants from healthy ones that show pseudo virus like symptoms (Pearson *et al*., 2006).

Although accurate identification of symptoms is necessary to describe the disease, in general speaking, it is often difficult to diagnose plant virus diseases merely by observing host symptoms. This is due to either of the following reasons including several unrelated viruses may induce similar symptoms in the same plant and same virus or its strains can result in different symptoms on the same and/or different host species, virus-like symptoms may also develop for physiological reasons and symptoms may be very slight and inconclusive or infected plants may not show clear symptom (Webster *et al*., 2004).

Because of the increased worldwide movement of germplasm through seed and other propagative material in global trade and agriculture, diagnosis of viruses in these materials assumes greater importance for national quarantine services to ensure the safe movement of germplasm across borders. Development of an efficient diagnostic procedure will enable to detect viruses during various stages of plant development as the infection usually remains in the host plant for lifetime (Alvarez, 2004).

Several diagnostic or detection methods are available for the identification of plant viruses, each with comparative advantages and disadvantages. For the development and selection of efficient and stable diagnostic methods, the following factors should be considered: (1) sensitivity, i.e. lower limits a virus may be detected, (2) accuracy and reproducibility, (3) number of samples that can be processed in a given time, (4) cost and sophistication of the apparatus and materials needed, (5) level of training required for operators (6) the amount of known information about the virus or disease and (7) adaptability to field conditions (Jan *et al*., 2011).

Quick identification of a plant virus species in a crop is pivotal for monitoring surveillance, vector association, and consequential ecological and epidemiological significance of the virus, vector and disease. All the techniques can broadly be classified into four major categories including biological, physical, serological and molecular approaches (Singh and Singh, 1995; Trigiano *et al*., 2008). In this section commonly used serological and molecular methods will briefly be described.

### 2.7.1 Serological Methods

#### 2.7.1.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a powerful method for detecting and quantifying a specific protein in a complex mixture. Originally described by Engvall and Perlmann (1971), the method enables analysis of protein samples immobilized in microplate wells using specific antibodies. ELISA was introduced to the study and identification of plant viruses in the 1970s (Voller *et al*., 1976; Clark and Adams, 1977). The ELISA technique relies on the basic principle in which the virus antigens are recognized by their specific antibodies (IgG) in association with colorimetric properties. The technique has revolutionized immunology and has commercial applications, including the detection of disease markers, allergens in the diagnostic and food industries. All ELISA techniques rely on the specific interaction between an epitope found on an antigen, and a matching antibody binding site. Although different approaches of ELISA have been developed, the direct and the indirect ELISA (Fig. 2.1) are the most commonly used methods for diagnosis of plant virus diseases and it is always recommended to include a homologous antigen (positive control) for the specific virus antibody and extracts from healthy plants as negative control so as to compare the absorption readings and to obtain a correct interpretation of results (Lima *et al*., 2012).

Two types of antibodies are used in ELISA; monoclonal (derived from unique antibody producing cells called hybridomas and capable of specific binding to a single unique epitope) or polyclonal (a pool of antibodies purified from animal sera that are capable of binding to multiple epitopes). Both monoclonals and polyclonals can be used in an ELISA. Polyclonals are more typically used for the secondary detection layer in indirect ELISA and monoclonal antibodies are more typically used for capture or primary detection of the antigen (Konstantinou, 2017; Lima *et al*., 2012).

There are four basic ELISA formats (Fig. 2.1), allowing for a certain amount of flexibility which can be adjusted based on the antibodies available, the results required, or the complexity of the samples. These include: Direct, Indirect, Sandwich (Capture) and Competitive (Inhibition) ELISA (Gan and Patel, 2013; www.bosterbio.com).

### Direct ELISA

For direct detection, an antigen coated to a multi-well plate is directly detected by an antibody that is conjugated to an enzyme.

Direct ELISA is simple and fast because only one antibody and fewer steps are used and no cross-reactivity of secondary antibody. Its drawbacks include high backround, labeling primary antibodies for each specific ELISA system is time-consuming and expensive, no flexibility in choice of primary antibody label from one experiment to another and minimal signal amplification (Engvall and Perlmann, 1971).

### Indirect ELISA

During the indirect or plate-trapped antigen (PTA) ELISA, the antigen coated to a plate or captured on a capture antibody is detected in two stages or layers. First an unlabeled primary antibody, which is specific for the antigen, is applied followed by bounding of an enzyme-labeled secondary antibody to the first antibody. The secondary antibody is usually an anti-species antibody and is often polyclonal. The indirect assay is the most popular format for ELISA (Konstantinou, 2017).

Indirect ELISA has many advantages such as a wide variety of labeled secondary antibodies are available commercially, versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection, it is sensitive because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification. As a drawback cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal and an extra incubation step is required in the procedure (www.bosterbio.com).

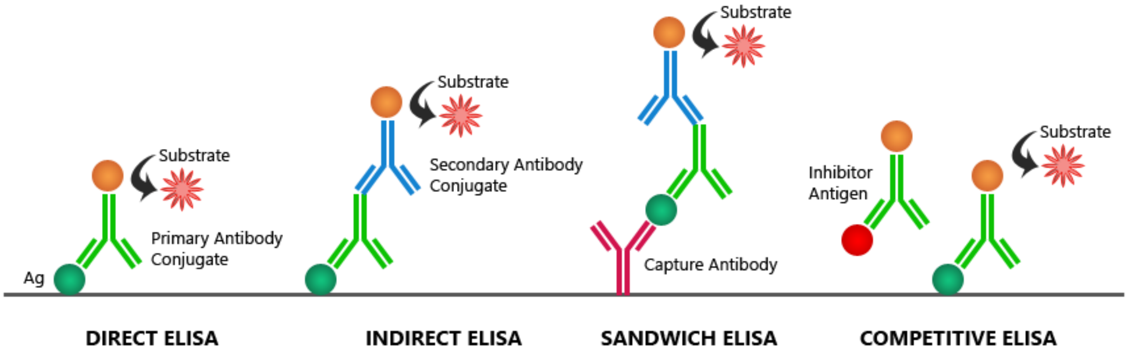


**Figure 2.1** Four most common ELISA formats. Source: www.bosterbio.com.

### Sandwich (Capture) ELISA

Sandwich ELISA typically requires the use of matched antibody pairs, where each antibody is specific for a different, non-overlapping part (epitope) of the antigen molecule. A first antibody (known as capture antibody) is coated to the wells. The sample solution is then added to the well. A second antibody (known as detection antibody) follows this step in order to measure the concentration of the sample. If the detection antibody is conjugated to an enzyme, then the assay is called a direct sandwich ELISA. If the detection antibody is unlabeled, then a second detection antibody will be needed resulting in an indirect sandwich ELISA (Clark and Adams, 1977).

The benefits of sandwich ELISA include high specificity: the antigen/analyte is specifically captured and detected; suitable for complex (or crude/impure) samples: the antigen does not require purification prior to measurement and flexibility and sensitivity: both direct and indirect detection methods can be used.

There are two commonly used sandwich ELISA formats (Fig. 2.2). These are Double Antibody Sandwich (DAS)-ELISA is a direct ELISA type in which the antigen is sandwiched between the capture and detection antibody (Clark and Adams, 1977). Triple Antibody Sandwich (TAS)-ELISA is an indirect type in which the antigen is sandwiched between a capture antibody on one side and similar antibody on the other side which in turn binds with detection antibody (Thomas *et al*., 1986). In TAS ELISA, the antibodies which directly interact with the virus are produced in one mammal (rabbit, for example) and an anti-rabbit IgGs are produced in a second animal species such as mice or goats. So, the detecting antibody conjugate binds specifically to the primary virus specific antibody (Lima *et al*., 2012).

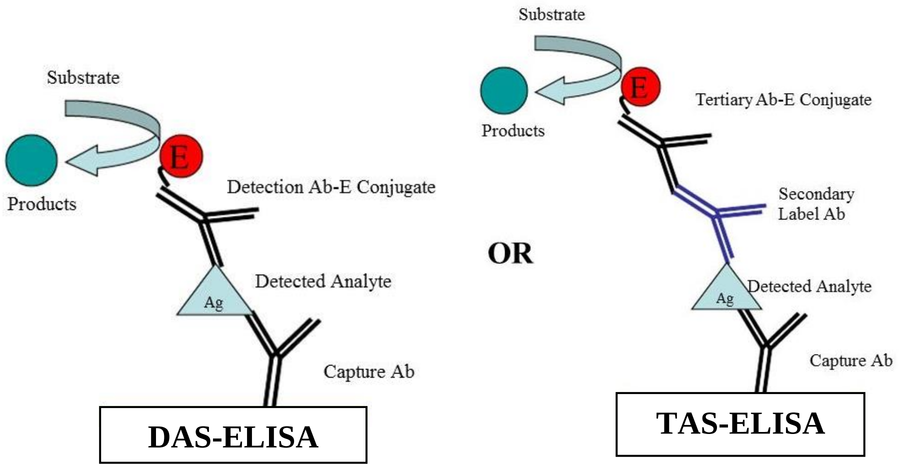


**Figure 2.2** DAS and TAS ELISA formats. Source: www.nanohub.org.

### Competitive or Inhibition ELISA

The key event of competitive ELISA is the process of competitive reaction between the sample antigen and antigen bound to the wells of a microtiter plate with the primary antibody. First, the primary antibody is incubated with the sample antigen and the resulting antibody-antigen complexes are added to wells that have been coated with the same antigen. After an incubation period, any unbound antibody is washed off. The more antigens in the sample, the more primary antibody will be bound to the sample antigen. Therefore, there will be a smaller amount of primary antibody available to bind to the antigen coated on the well, resulting in a signal reduction. The main advantage of this type of ELISA arises from its high sensitivity to compositional differences in complex antigen mixtures (Dobrovolskaia *et al*., 2006).

It has high specificity; since two antibodies are used the antigen/analyte is specifically captured and detected even when the specific detecting antibody is present in relatively small amounts. It is also **s**uitable for complex samples, since the antigen does not require purification prior to measurement. Moreover it is flexibility and sensitivity, since both direct and indirect detection methods can be used.

In the above mentioned ELISA formats, the antigen is detected either directly (enzyme-labeled primary antibody) or indirectly (enzyme-labeled secondary antibody). The detection antibodies are usually conjugated either with alkaline phosphatase (AP), horseradish peroxidase (HRP), β-D-galactosidase or β-lactamase, of which, AP and HRP are most commonly used. A large selection of substrates is available for performing the

ELISA with these antibody conjugated enzymes. The choice of substrate depends upon the required assay sensitivity and the instrumentation available for signal detection (spectrophotometer, fluorometer or luminometer). Enzymatic signal generation requires the catalysis of a substrate to produce a colored or fluorescent compound or chemiluminescence (visible light). The signal is measured using a spectrophotometric plate reader, a fluorometer with the appropriate filters or a luminometer set to read total light output (Dobrovolskaia *et al*., 2006; www.bosterbio.com).

Colorimetric substrates form a soluble, colored product that accumulates over time relative to the amount of enzyme present in each well. When the desired color intensity is reached, the product absorbance is either measured directly or in some cases a stop solution is added to provide a fixed end point for the assay. Colorimetric substrates are available for both horseradish peroxidase (3,3',5,5'-Tetramethylbenzidine (TMB), o-phenylenediamine dihydrochloride (OPD), 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)) and alkaline phosphatase; p-nitrophenyl phosphate (pNPP). Chemifluorescent detection is also enzyme-based but the generated product is fluorescent rather than colorimetric. The signal is measured using a fluorometer with the appropriate excitation and emission filters. Chemifluorescence reactions are either measured over time in kinetic assays or halted using a stop solution for direct measurement (Konstantinou, 2017; www.bosterbio.com).

Chemiluminescence is a chemical reaction that generates energy released in the form of light. Most chemiluminescent substrates are HRP-dependent although some AP equivalents are available. The most common approach is to use luminol in the presence of HRP and a peroxide buffer. The luminol is oxidized and forms an excited state product that emits light as it decays to the ground state. Light emission occurs only during the enzyme-substrate reaction, therefore when the substrate becomes exhausted the signal ceases. Chemiluminescent detection is generally considered to be more sensitive than colorimeteric detection (Konstantinou, 2017).

In a typical ELISA technique first the appropriate concentration of antibodies are coated to each well in duplicate or triplicate for reproducibility within a microtiter plate (sorbent). After two to four hours of incubation at 37 °C by sealing the plate, unbound antibodies will be removed by washing to prevent unspecific reactions. A sap, extracted from the infected plant tissue with appropriate buffer is added to the wells of the coated plates together with controls, known infected plants (positive control) and extracts from healthy plants (negative control). If the virus is available in the soap extract, it will bind to the antibodies fixed on the wells of the plate. After incubation, this will be followed by washing step to remove unbound extract, which otherwise may interfere with the final result. Binding of the virus and specific antibody is made visible through an antibody conjugated with an enzyme (usually AP). Addition of substrate to the conjugated AP induces a colorimetric reaction, that is, conversion of the chromogenic substrate *p*-nitrophenyl phosphate (colorless) to a colored, water soluble product, *p*-nitrophenol (yellow in alkaline solution) (Voller *et al*., 1976; Clark and Adams, 1977).

The color change is detected by a calibrated microtitre plate spectrophotometer at 405 nm wave length which indirectly tells the presence of the virus in the assayed sample and the negative wells will be colorless. It is critical that positive and negative controls are included in each assay to define a threshold for differentiating between “infected” and “non-infected” samples. Generally, a sample is regarded as positive if the absorbance value exceeds the mean value of a negative control by 2-3 standard deviations. In some cases, the simple arithmetic cut-off of twice the absorbance value of the average of the negative controls is used (Clark and Adams, 1977; Torrance, 1998; Crowther, 2001; Webster *et al*., 2004; Lima *et al*., 2012).

A modified form of ELISA called voltametric enzyme immunoassay, detects the change in electric conductivity of the substrate after being acted upon by the conjugate enzyme in place of color change. This method is reported to be more sensitive than ELISA. It has been used to detect *Cucumber mosaic virus* (Sun *et al*., 2001).

#### 2.7.1.2 Dot-Blot Immuno Binding Assay (DBIBA)

In dot-blot immune binding assay, a membrane (nitrocellulose or nylon) or even plain paper is used as the solid substrate for ELISA test. At the initial step the virus in a plant extract is dot-blotted on to the membrane. The sample application on the membrane is usually accomplished through the use of a plastic mold with 96 wells which presses the membrane marking the places where the samples should be applied. Usually the spaces not occupied by the antigens on the membrane are blocked with neutral protein solution. The addition of virus IgG produced in rabbit and the anti-rabbit IgG produced in mouse follow protocols similar to indirect ELISA, except that the positive reactions in DBIBA are recorded as colored dots on the membrane. As DBIBA is a simple, less laborious and quick test, it can be used routinely for plant virus indexing and survey programs. One disadvantage of DBIBA is the possibility of sap components interfering with the antigen-antibody reactions, resulting in subsequent problems with the diagnostic results (Astier *et al*., 2007).

Then a particular substrate is added which the enzyme conjugated with the specific IgG converts it to an insoluble colored material. Dot blot ELISA tends to be rapid, easy to perform and conserve reagents and often more sensitive than ELISA carried out in a microtitre plate (Banttari and Goodwin, 1985).

Despite their sensitivity, serological methods which rely on the production of virus-specific antibodies cannot be used for virus and viroids detection of unknown origin. Moreover, ELISA test often fails because of low virus titre and due to inhibitory effect of the compounds present in the cell sap of plants (Jan *et al*., 2011).

#### 2.7.1.3 Immune Precipitation ELISA (IP- ELISA)

This method is used for plant viruses whose particles are not well adsorbed in the ELISA plate wells and involves precipitation of the immune virus particles with specific antiserum. This will be achieved first by grinding a sample with appropriate extraction buffer and mixing the sap with equal volume of diluted antibody. The mixture will be incubated at 37 °C for 3 hours or overnight at 4 °C and centrifuged. The pellet containing the virus particles linked to the antibodies are re-suspended in extraction buffer and used as conventional indirect ELISA. IP-ELISA was shown to be a sensitive and practical diagnostic technique for plant viruses whose virus particles do not adsorb well on the plate wells (Lima *et al*., 2011).

#### 2.7.1.4 Tissue-Blot Immuno Assay (TBIA)

This method employs use of antibodies raised against specific viruses like that of ELISA. The sap extract from infected plant tissue is expressed on to blotting paper; nitrocellulose or nylon membranes and the virus is identified by labeled chemiluminescent probes. As compared to ELISA it is relatively simple, rapid, sensitive, cheap and less labour-intensive. It is suitable method for surveying large number of sample (Lin *et al*., 1990; Webster *et al*., 2004).

#### 2.7.1.5 Immunosorbent Electron Microscopy (ISEM)

Immunosorbent Electron Microscopy (ISEM) combines the specificity of serological assays with the visualization capabilities of the EM. Virus particles are selectively “trapped” on antibody-coated grids with little contaminating host-plant material. Hence, the technique is more sensitive for detecting viruses than the leaf dip method. In addition to diagnosis, ISEM can also be used to estimate the degree of serological relationship between viruses. Although ISEM is sensitive technique, it has the same drawbacks as EM. Nonetheless, it is ideal for confirmatory tests using small numbers of samples, if the EM facility and specific antisera are available (Roberts and Harrison, 1979; Milne, 1991).

#### 2.7.1.6 Quartz Crystal Microbalance Immunosensors (QCMI)

The QCM measures mass based on vibrations and frequency change in real time and it has been frequently used to measure small mass in vacuum, gas and liquid conditions (Kurosawa *et al*., 2006). Immunological combination with QCM results in QCMI as a mass-sensitive transducer device. Antigen-antibody binding reaction causes decreased quartz crystal oscillation frequency in positive reaction (Owen *et al*., 2007).

To proceed with this technique, a quartz crystal disk is coated with virus-specific antibodies. Voltage is applied across the disk, making the disk warp slightly via a piezoelectric effect. Adsorption of virus particles to the crystal surface changes its resonance oscillation frequency in a concentration-dependent manner. This method is economical and equally sensitive but more rapid than ELISA (Chen and Tang, 2007). In the first described use of QCM for plant viruses, as little as 1ng of particles of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* were detected in crude sap extracts (Eun *et al*., 2002). The detection instrument for QCM is portable and QCM coated with virus-specific antibodies to detect plant viruses has long life span, hence, it can be used for onsite detection of plant viruses (Eun *et al*., 2002; Becker and Cooper, 2011).

#### 2.7.1.7 Lateral Flow Assay (LFA)

Lateral flow assays (LFAs), are fast serological methods with prefabricated strips of a carrier material containing dry reagents that are activated by applying a fluid sample. They are used (i) in phytopathology, medical and veterinary practice aimed to indicate the cause of an infection, ascertain pregnancy, or failure of internal organs (Eg., heart attack, renal failure or diabetes); (ii) to ascertain the safety of food and feed or the environment for presence of toxic compounds or allergens; (iii) in forensic studies, including abuse of (illicit) drugs (Miller, 2015; Koczula and Gallotta, 2016).

Lateral flow assay works based on the principle of immunochromatography where the response of the reaction is determined by the specific interaction of antibody and antigen (Fig. 2.3). LFAs are user-friendly diagnostic techniques especially designed for field survey on site detection outside the laboratory. For virus detection, strips containing virus-specific antibodies are placed in extracts from plants to be assayed, and the sample wicks upward. If virus is present, it reacts with specific antibody to give a visible band at the test line on the strip, which indicates a positive result (Ward *et al*., 2004).

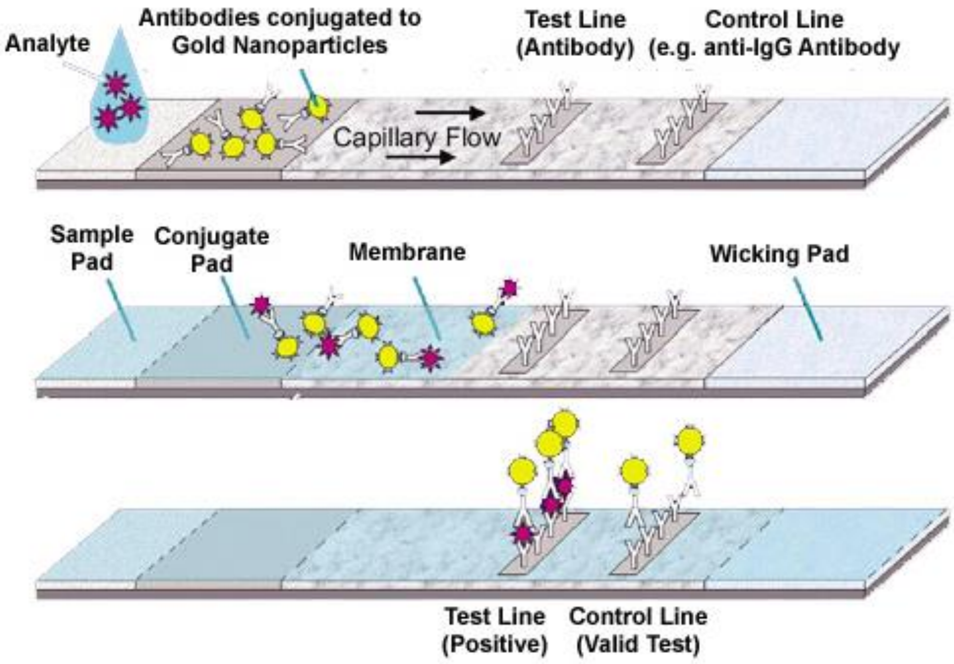


**Figure 2.3** Architectural description of Lateral flow assay. Source: Miller, 2015.

### 2.7.2 Molecular Methods used in plant viral disease detection

Nucleic acid-based methods, based on amplification or hybridization, are sensitive and specific which allow genetic relationships to be determined, have revolutionized the method of plant virus detection and identification. Specificity is directly related both to design primers or probes and to the amplification or hybridization protocols (Jan *et al*., 2011).

#### 2.7.2.1 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Conventional PCR

Reverse transcriptase (RT)-PCR, which passes through cDNA synthesis stage, is a standard method for the detection of RNA viruses as that of PCR which is common method for DNA viruses. RT-PCR is extremely sensitive, fairly inexpensive and requires technical skill (Lotos *et al*., 2014). As initial knowledge of the nucleotide sequence is required in order to design oligonucleotide primers, it cannot be used in identifying an unknown virus (Webster *et al*., 2004).

#### 2.7.2.2 Multiplex PCR

Multiplex PCR is helpful for simultaneous detection of multiple species or strains of different DNA and RNA viruses infecting a single host. It uses multiple primer sets, each one targeting a particular pathogen. To avoid cross reactivity, the amplicons to be amplified are of different length (Nie and Singh, 2000; Lopez *et al*., 2009). This method was used to detect one virus, *Apple stem grooving virus* and six viroids; *Citrus exocortis viroid*, *Citrus bent leaf viroid*, *Hop stunt viroid*, *Citrus viroid III*, *Citrus viroid IV* and

*Citrus viroid OS* from citrus plants in a single reaction tube (Ito *et al*., 2002). Five major apple viruses including *Apple mosaic virus*, *Apple stem pitting virus*, *Apple stem grooving virus*, *Apple chlorotic leaf spot virus* and a single viroid, *Apple scar skin viroid* were identified in a single reaction (Kumar *et al*., 2014). Zhang *et al*. (2016) have also reported the simultaneous detection of three distinct genotypes (BrYV-A, BrYV-B and BrYV-C) of a new polerovirus species, *Brassica yellows virus* (BrYV). This method was also employed for the detection of two morphologically and genomically dissimilar viruses, *Rice tungro bacilliform virus*, a dsDNA virus and *Rice tungro spherical virus*, a single stranded RNA virus causing Rice tungro, economically the most important viral disease of rice (Periasamy *et al*., 2006). Since more than one amplicone is amplified in a single tube reaction, multiplex PCR is cost effective method and saves time.

#### 2.7.2.3 Immunocapture- RT- PCR (IC- RT- PCR)

Immunocapture RT-PCR is used with plant extract or with immobilized targets, allowing detection of minimal quantities of RNA targets from plant material or insect vectors without going for extract preparation. IC-PCR combines capturing of virus particles by antibodies followed by PCR amplification, that is, serology and PCR (Nolasco *et al*., 1993). In IC-PCR, first, microtiter tubes are coated with specific virus antibodies and incubated at 37 °C for 2 h. After washing, the microtiter tubes coated with the antibodies will trap the virus particles and then removed by heating with a nonionic surfactant (such as Triton X-100) followed by PCR. It is useful in concentrating virus particles where virus titre is low or exists in variable concentration or where compounds that inhibit RT-PCR reaction are present in the plant extract (Hema *et al*., 2003; Mulholland, 2009). This method shows increased detection sensitivity (105 times) than ELISA (Sano *et al*., 1992; Wetzel *et al*., 1992). This method was used for RNA amplification of five virus species of the genera *Comovirus*, *Cucumovirus*, *Potyvirus* and *Sobemovirus* from different infected plant tissues. ICRT-PCR is practical, sensitive and minimized problems with total RNA extractions from infected tissues (Lima *et al*., 2014).

#### 2.7.2.4 Nested PCR

Nested PCR involves a one-step PCR, in which two sets of primers are used in two successive reactions. In the first PCR, one pair of primer is used to generate DNA segments which will be used as a target for the second reaction. This is used to increase the specificity of target DNA amplification. The second pairs of primers, in which their binding sites are located (nested) within the first amplified fragment, amplify the desired gene in the second cycle of amplification. This increases specificity and sensitivity of detection which is important for the detection of pathogens that occur in very few amounts (Webster *et al*., 2004; Lopez *et al*., 2009). It has been applied to detect *Lettuce mosaic virus* even from a single aphid (Moreno *et al*., 2007). Nested PCR can be combined with IC-RT PCR to increase sensitivity and to simplify preparation of sample (Helguera *et al*., 2001, 2002).

#### 2.7.2.5 Double Stranded RNA Extraction for Virus Identification

Double stranded RNAs (dsRNAs) are formed during virus replication and consist of full-length genomic, sub-genomic and defective RNAs of single stranded RNA (ssRNA) viruses or genomes of dsRNA viruses. Most plant viruses have positive, ssRNA genomes that are replicated within the cytoplasm of the host cell. Viral RNA-dependent RNA polymerases (vRdRPs) synthesize complementary negative stranded genomic RNA, from which numerous copies of positive stranded RNA are reproduced. Partial or complete annealing of positive and negative RNA trands constitutes the replicative form (RF), which provides one source of dsRNA (Voinnet, 2005).

Isolation of dsRNA from virus infected plant tissue is based on the affinity of cellulose powder for nucleic acids, specifically, the adsorption of dsRNA at 15% ethanol concentration followed by repeated washing of the cellulose with buffer/ ethanol, finally elution of the dsRNA in buffer without ethanol. There is often some contamination of the purified dsRNA with DNA or single-stranded RNA which can be hydrolyzed using DNAse and RNAse under high ionic strength conditions, respectively. The resulting sample can then be bound to cellulose again in the presence of ethanol, washed, and eluted. The eluted dsRNA is precipitated and an aliquot used for analysis on an agarose or polyacrylamide gel (Morris and Dodds, 1979).

The sizes and patterns of these virus-specific dsRNAs after separation on gels can be useful in virus characterization and provide useful information on the type of virus(s) that may be infecting a plant. The usefulness of dsRNAs from virus infected plants is based on the premise that healthy plants not infected with a virus do not contain dsRNAs. The presence of multiple dsRNA bands on agarose or polyacrylamide gels may indicate the presence of a monopartite virus that produces sub-genomic RNAs during its replication cycle or a multipartite virus that replicates via a polyprotein strategy or it may be an indication that the plant is infected with more than one virus (Voinnet, 2005).

### 2.7.2.6 Isothermal Amplification Methods

Isothermal amplification is a method of nucleic acid amplification carried out at a constant temperature without thermocycling required in PCR. Like PCR, extension of target-specific primers is catalyzed by a DNA polymerase (sometimes RNA polymerase). The challenge of isothermal amplification is, therefore, to enable primer binding, such that amplification can occur without the repeated cycles of denaturation and annealing PCR (Deng and Gao, 2015).

Single-stranded primer binding sites can be generated without thermal cycling by either of the following methods including: non-thermal methods of template denaturation, transcription of RNA, strand displacement around a circular template, nicking or partial degradation of primer extension products to allow extension or further rounds of priming and formation of secondary structure containing single stranded primer binding sites (Deng and Gao, 2015).

There are different types of established isothermal amplification methods. These include: helicase dependent amplification (HDA), nucleic acid sequence based amplification (NASBA), recombinase polymerase amplification (RPA), rolling circle amplification (RCA), isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) and loop-mediated isothermal amplification (LAMP) (Deng and Gao, 2015).

### *2.7.2.6.1 Helicase Dependent Amplification (HDA)*

Helicase dependent amplification is conceptually simple approach in which isothermal amplification of DNA is achieved by separating the double strands of template DNA by non-thermal means using helicase enzyme. This allows primer binding and extension by DNA polymerase at a constant temperature of approximately 65 °C. HDA generally may last for 30 to 90 min. This method sustains amplification of relatively short fragments of approximately 70-120 bp. It can be performed at a fixed temperature, but the inclusion of a brief incubation at 95 °C prior to the addition of helicase enzymes has been shown to increase sensitivity (Vincent *et al*., 2004).

### *2.7.2.6.2 Recombinase Polymerase Amplification (RPA)*

RPA uses a recombinase enzyme which forms a complex with primers to initiate amplification without thermal denaturation. This method doesn’t require an initial denaturation step but agitating after 5 min of incubation increases sensitivity of the reaction. The reaction is incubated at a low reaction temperature, 37 and 42 °C, which can easily be sustained by a low power instrument. However, the use of a low reaction temperature can result in the generation of more non-specific amplification artifacts than are typically observed in isothermal amplification methods, which use higher reaction temperatures. The major advantage of RPA is its short reaction times, which is typically < 30 min (Piepenburg *et al*., 2006).

### *2.7.2.6.3 Nucleic Acid Sequence Based Amplification (NASBA)*

It is a method for isothermal amplification of RNA based on transcription (Compton, 1991). A modified primer is used to incorporate the sequence of the T7 RNA polymerase promoter into a dsDNA intermediate, functionalizing the promoter and resulting in transcription of a ssRNA product at a reaction temperature of 41 °C. NASBA has been used for the detection of a number of plant pathogens in conjunction with molecular beacon probes, in a format sometimes referred to as AmpliDet. This format, in which fluorescence is monitored in real time to detect hybridization of the probe to the single-stranded amplicon, is a closed tube system and allows quantification of the target sequence, but requires the use of an instrument with real-time fluorescence monitoring capability (Leone *et al*., 1997; Klerks *et al*., 2001).

NASBA requires denaturation of the template to allow primer annealing prior to the addition of non-thermo-stable enzymes, making reaction setup a two stage process. NASBA is considered to be a highly sensitive detection method but with relatively long reaction times, usually 90 min. Both NASBA and HDA perform optimally with a two-step thermal profile and as such are not truly isothermal. RPA additionally requires the tubes to be manually agitated, after several minutes of amplification, to achieve optimum performance. Each of these characteristics makes these chemistries less suitable for onsite use, increasing the time and complexity of amplification (Klerks *et al*., 2001).

### *2.7.2.6.4 Rolling Circle Amplification (RCA)*

RCA is used to amplify circular nucleic acids utilizing the strand displacement activity of Phi29 DNA polymerase after amplification with hexamer degenerate primers. RCA followed by restriction fragment length polymorphism (RFLP) analysis has been used for diagnosis of geminiviruses which have small circular ssDNA genomes (Haible *et al*., 2006).

### *2.7.2.6.5 Isothermal and Chimeric Primer-initiated Amplification of Nucleic acids (ICAN)*

Isothermal and Chimeric primer-initiated Amplification of Nucleic acids (ICAN) method amplifies target DNA under isothermal conditions around 55 °C using a pair of chimeric (5’-DNA-RNA-3’) primers with a thermostable RNaseH which introduces a nick at the junction between the DNA and RNA portions of the primers, and a DNA polymerase with strand displacing activity which continues extension from the nick site (Mukai *et al*., 2007).

### *2.7.2.6.6 Loop Mediated Isothermal Amplification (LAMP)*

The isothermal amplification methods discussed so far each have various advantages. However, in the context of developing methods for on-site use, factors such as reaction time (> 1 h in the case of HDA, NASBA, and ICAN) and complexity of assay design (RPA, RCA for non-circular targets) are potential disadvantages. An alternative isothermal amplification approach is to design primers such that the amplification products contain single stranded primer binding sites. LAMP is the most commonly used method to take this approach, using a polymerase with high strand displacement activity besides the replication and four or six primers which recognize a total of six specific sites on the target DNA to generate an amplification product. This product contains single stranded loop regions to which primers can bind without template denaturation at a reaction temperature of around 60 - 65 °C. The LAMP process is initiated by an inner primer containing sequences of the sense and anti-sense strands of the target DNA (Notomi *et al*., 2000).

The internal primers introduce self-complementarity into the amplification product, causing loops formation, while extension of the external primers causes displacement of the single stranded extension products of the internal primers. The products of LAMP reactions consist of alternately oriented repeats of the target sequence. The addition of loop primers accelerates amplification by priming at the loop regions that are of the incorrect orientation for the internal primers to bind. Loop primers increase sensitivity and reduce reaction times. However, to accommodate loop primers requires a longer region of suitable sequence, such that design of two loop primers may not be possible, and many assays have been reported in the literature, which achieve acceptable performance without loop primers, or with only one loop primer. A modification of the LAMP reaction incorporates one or more ‘stem’ primers, which bind to the double stranded central portion of each repeat of the amplified region, to further enhance assay performance and increase primer design options (Gandelman *et al*., 2011).

As LAMP uses at least six primer binding regions, it is possible to design assays with high specificity by positioning each primer at the site of mismatches between the target and non-target species (Fig. 2.4). LAMP assays have been reported with sensitivity approaching that of comparable real-time PCR assays and typically exceeding that of conventional PCR (Tomlinson *et al*., 2007). LAMP does not require initial template denaturation and more recently developed strand displacing DNA polymerases display faster reaction kinetics, such that LAMP reaction times can be reduced to < 30 min. LAMP, in common with other isothermal DNA amplification methods, can be modified for detection of RNA targets by the addition of reverse transcriptase to the reaction. In RT-LAMP, reverse transcription and amplification of cDNA proceed concurrently at a single temperature of around 65 °C. Significantly for onsite testing, LAMP has been reported to be tolerant of some substances which are inhibitory to PCR potentially allowing LAMP to be used in conjunction with simplified nucleic acid extraction methods (Kaneko *et al*., 2007).

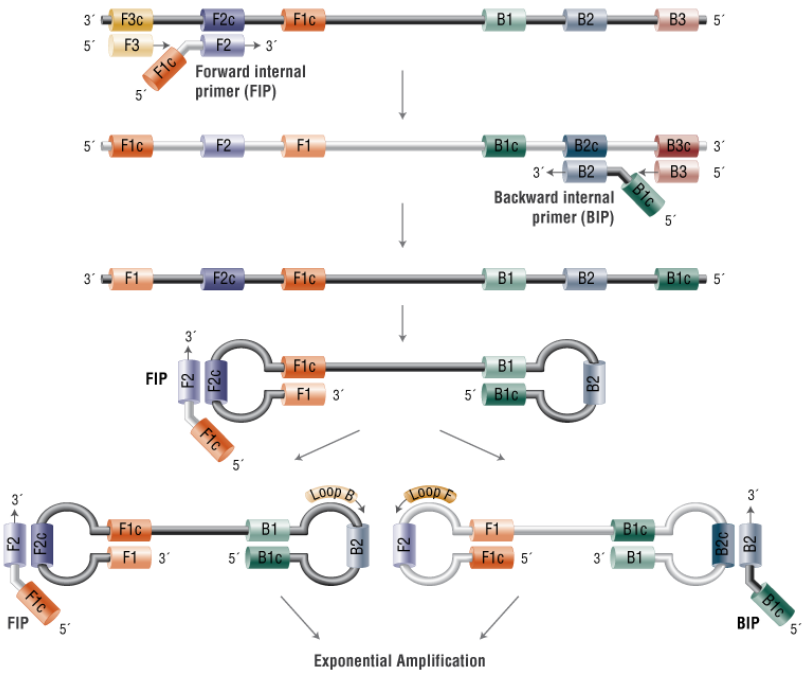


**Figure 2.4** Loop-mediated isothermal amplification (LAMP) uses 4-6 primers recognizing 6-8 distinct regions of target DNA. A strand-displacing DNA polymerase initiates synthesis and 2 of the primers form loop structures to facilitate subsequent rounds of amplification. Source: www.neb.com, 2016.

### 2.7.2.7 Quantitative Real Time PCR

Conventional PCR (cPCR) has emerged as a main tool for the diagnosis of plant pathogens and has contributed to reducing some problems related to the plant pathogens detection (Martin *et al*., 2000). On the other hand, because of different testing parameters in cPCR assays, optimization of conditions is challenging and time consuming (Espy *et al*., 2006).

The necessity of fast, sensitive, and specific methods to detect pathogens is important to improve decision making in plant viral disease control (Lievens *et al*., 2005). Quantitative real-time PCR (qPCR) technology allows accurate detection and/or quantification of viruses, which cannot be extracted or cultured easily from host tissue, or are presented at low inoculum load in host plant samples. Real time PCR works based on the detection of the fluorescence produced by a reporter molecule which increases as the reaction proceeds in real time. There are two common methods for the detection of products in real-time PCR: 1) non-specific fluorescent dyes that intercalate with any dsDNA; and 2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence (Garrido *et al*., 2012).

Real-time PCR has two main important features compared to cPCR. The first one, it saves time, data are available in real time, on screen, do not require post-PCR processing (Eg., electrophoresis, colorimetric reaction or hybridization). Secondly, it commonly amplifies short DNA fragments (70-100bp), which favors a higher level of efficiency and sensitivity (Garrido *et al*., 2009).

#### *2.7.2.7.1 Detection Based on Non-specific Label Method*

*SYBR Green Dye*

In real-time PCR assays, DNA binding dyes are utilized as fluorescent reporters to monitor the reaction. As the PCR product accumulates with each consecutive cycle of amplification, the fluorescence of reporter dye is enhanced. Therefore, it is possible to monitor the PCR reaction during the exponential phase by recording the amount of fluorescence emission at each cycle. SYBR Green, most widely used for real-time PCR assays, is an intercalating dye which binds to a minor groove of dsDNA. It does not emit fluorescence signal, unless it binds to the dsDNA. Its application is relatively cheaper than other detection methods. However, the principal drawback with SYBR green is that it is not specific and it intercalates with non-specific products. So, the formation of non-specific amplicons can lead to false positive results in the quantification (Giulietti *et al*., 2001).

#### *2.7.2.7.2 Detection Based on Sequence Specific Methods*

##### *A. TaqMan Probe Based Detection*

TaqMan probes are hydrolysis probes which are labeled on both sides and utilize the 5' exonuclease activity of the Taq DNA polymerase for measuring the amount of target sequences. TaqMan probes consist of a sequence of 25-30 nucleotides which is labeled with a donor fluorophore (as reporter) at the 5' end, and an acceptor dye (as quencher) at the 3' end. Generally, a fluorophore is a molecule that absorbs light energy and is promoted to an excited state, and a quencher is a molecule that can receive energy from a fluorophore and disperse the energy by proximal quenching or by fluorescence resonance energy transfer (FRET) (Didenko, 2001; Schena *et al*., 2013).

In FRET quenching, as a dynamic quenching mechanism, the fluorophore transfers its energy to the quencher, and the energy is released as light of a longer wavelength. So, until the hydrolysis of the probe, the quencher and the fluorophore are close to each other, separated by the probe length. However, this proximity does not entirely quench the fluorescence of the reporter dye and background fluorescence is detected. During PCR, the probe hybridizes to the single-stranded DNA (ssDNA) template. In the extension step, the probe is cleaved by the 5'-nuclease activity of Taq DNA polymerase when the enzyme reaches the probe, resulting in the separation of the fluorescent reporter dye from the quencher, thus generating a fluorescent signal. The fluorescence intensity is therefore a direct consequence of the amplification process (Didenko, 2001; Schena *et al*., 2013).

In proximal quenching, unlike FRET, while the probe is intact, due to close proximity between them, the quencher absorbs the energy from the reporter dye and dissipates as heat. As a result, no fluorescence is recognized. One advantage of the TaqMan probes over SYBR Green dye is that their specificity. Specific hybridization between probe and target DNA sequence is required to produce fluorescent signal. A TaqMan real-time PCR assay can be also multiplexed to detect several distinct sequences in a single PCR reaction tube due to possibility of labeling of the fluorogenic probes with different detectable reporter dyes (Schena *et al*., 2004). However, the labeling of TaqMan probe with double dyes and its designing is more complicated than in SYBR Green primers, making this assay more expensive than SYBR Green assay (Okubara *et al*., 2005).

### *B. Molecular Beacon Based Detection*

Molecular beacons are single stranded oligonucleotide hybridization probes which form a stem and loop (hairpin) structure. The loop of the probe is complementary to the target sequence and its two ends are complementary to each other. A fluorophore is tagged at the 5' end of the probe and a quencher at the 3' end. When the probe sequence in the loop anneals to a complementary nucleic acid target sequence, the stem portion of the beacon separates out and hybridizes to the target, resulting in the fluorescence emission. In the absence of a complementary target sequence, the beacon remains closed and there is no appreciable fluorescence. Fluorescence is screened during each annealing step when the beacon is attached to its complementary target. So, the amount of fluorescence at each cycle depends on the amount of specific product. This method can be used to develop extremely specific assays that other types of probes could not achieve (Schena *et al*., 2004).

### *C. Scorpion Probe Based Detection*

Scorpion primers are bi-functional molecules in which a primer is covalently linked to a specific probe sequence that is held in a hairpin-loop form with a fluorophore at one end and a quencher at the other. At the 5'- end, the Scorpion primer sequence contains a non-target sequence as PCR blocker at the start of the hairpin loop that prevents polymerase read through. This structure brings the fluorophore in close proximity with the quencher and avoids fluorescence. Scorpion makes the molecular beacon technique more efficient by combining the functions of the probe and the 5' PCR primer (Didenko, 2001).

In the absence of the target, the quencher nearly absorbs the fluorescence emitted by the fluorophore. As soon as annealing between the primer-probe and the target occurs, scorpion primer combines to the PCR product and then the probe sequence in the tail curls back to hybridize with the target sequence. As the tail of the scorpion and the amplicon are part of the same strand of DNA, the interaction is intra-molecular (Giulietti *et al*., 2001). In Scorpion primers, the probe is physically coupled to the primer which means that the reaction leading to signal generation is a unimolecular which is efficiently instantaneous (Tomlinson *et al*., 2007). This leads to stronger signals, more reliable probe design, enhanced discrimination, and shorter reaction times compared with molecular beacons and TaqMan probes. Relative sensitivities of the real-time PCR techniques in increasing order are: SYBR Green < TaqMan < Molecular beacons < Scorpion. Of course, one should bear in mind that probe based techniques reveal a greater dynamic range than SYBR Green chemistry (Okubara *et al*., 2005).

#### 2.7.2.8 Next Generation Sequencing (NGS) in Viral Diagnostics

The introduction of next (second) generation sequencing (NGS) technologies has led to a revolution in virus discovery and exciting new possibilities for diagnostics; the application of massively parallel sequencing approaches, and subsequent bioinformatics analysis for viral sequences, carries the promise of routine, generic detection of viruses and other pathogens. A number of approaches were reported applying NGS to identify diverse plant viruses, using different sequencing platforms and nucleic acid preparations as starting material (Adams *et al*., 2009; Al Rwahnih *et al*., 2009; Kreuze *et al*., 2009) and many others have since followed.

A metagenomic approach to diagnostic plant viruses offers the possibility of overcoming the problems of pathogen prediction associated with parallel screening methods and the non-specificity associated with traditional investigational techniques. Sequences produced from an infected plant will include sequences from any pathogens present. The extraction of RNA from the infected plant, synthesis of complementary DNA (cDNA) with a random priming method followed by sequencing will produce sequences from a large range of potential pathogens. RNA viruses, viroids and the RNA stages of actively replicating DNA viruses can be directly sequenced. This approach should also produce sequences of mRNA and rRNA from any phytoplasma, bacteria or fungi present in the sample. By sequencing cDNA and not genomic DNA, only active host genes and ribosomes will be sequenced, avoiding the large amounts of untranscribed genomic DNA found in higher plants, and also avoiding integrated genomes of some plant viruses, such as badnaviruses (Adams *et al*., 2009).

Based on whether they employ the PCR amplification or not, there are two main categories of NGS technologies. The first four commercially available PCR-based platforms include: Roche 454 Genome Sequencer (Roche Diagnostics Corp., Branford, CT, USA), HiSeq 2000 (Illumina Inc., San Diego, CA, USA), AB SOLiD System (Life Technologies Corp., Carlsbad, CA, USA) and Ion Personal Genome Machine (Life Technologies, South San Francisco, CA, USA). The other category which lacks the PCR stage prior to sequencing involves ‘single molecule’ sequencing (SMS) technologies. Two SMS systems have been announced. These are HeliScope (Helicos Bio- Sciences Corp., Cambridge, MA, USA) and PacBio RSSMRT system (Pacific Biosciences, Menlo Park, CA, USA) (Shokralla *et al*., 2012).

#### 2.7.2.9 Third Generation Sequencing

Third generation sequencing is also called long-read sequencing. There are three commercially available third-generation DNA sequencing technologies. These are Pacific

Biosciences (PacBio) Single Molecule Real Time (SMRT) sequencing (Eid *et al*., 2009), the Illumina Tru-seq Synthetic Long-Read technology and the Oxford Technologies sequencing platform using Nanopores (Branton *et al*., 2008). Contrasting to second-generation , third generation sequencing technologies use single-molecule sequencing or clonal amplification for sequencing library preparation of long molecules and can produce long reads averaging between 5,000 bp to 15,000 bp, with some reads exceeding 100,000 bp. This capability to produce substantially longer reads from single DNA molecule is helpful to reduce numerous computational challenges and errors during second generation sequencing (Bleidorn *et al*., 2016; Lee *et al*., 2016).

Single Molecule Real-Time (SMRT) sequencing is based on monitoring polymerase activity while incorporating differently labelled nucleotides into the growing DNA strand. Each nucleotide carries a base-specific fluorescent label on its phosphate group, which is released when being incorporated by the polymerase. Incorporated nucleotides are detected by real-time imaging during strand synthesis. The whole process takes place in a small well which is surrounded by aluminium walls called Zero-mode waveguide (ZMW) (Eid *et al*., 2009).

Single DNA polymerase molecules are attached on the surface of these wells, where their activity can be monitored. With a diameter of 70 nm and a depth of 100 nm these wells are extremely small and ~150,000 ZMWs are comprised on so-called SMRT-cells for sequencing. For the sequencing process, fluorescent labelled nucleotides are flooded into these small cavities and their presence while floating in and out is measured as background noise. However, whenever a labelled nucleotide is associated with the template DNA in the polymerase active site a pulse of fluorescence intensity can be recorded for the corresponding dye. Such an emission of light lasts some milliseconds and will be detected by the ZMW sensor (Eid *et al*., 2009).

After incorporation, the fluorescent label is cleaved away by the normal activity of the polymerase, leading to a diffusion of the dye in the background and subsequent drop of the emission signal. With this technique, approximately 2-4 nucleotides are synthesized per second. The emission spectra not only help to unravel the DNA sequence of the template molecule, but also reveal possible epigenetic modifications due to subtle differences in emission patterns (Flusberg *et al*., 2010). The sequencing process is rather fast and takes about 4 hours per SMRT-cell. Reads are considerably longer than those of second generation sequencers, averaging more than 10 kbp, including reads as long as 54 kbp (Lee *et al*., 2014). However, this technique has high error rate, which is estimated to be up to 20% and the output per SMRT-cell is, at ~ 400 mbp, also relatively small (Hackl *et al*., 2014).

Illumina TruSeq Synthetic Long Reads was initially introduced in 2012, as the Moleculo protocol (Kuleshov *et al*., 2014). Using this approach, ~10 kbp molecules of DNA are clonally amplified and barcoded before sequencing with a short read instrument, so that long reads can be synthetically created from the short read sequences. The synthetic long reads are very accurate (~ 0.1% error) and can be used for phasing analyses and assembly without error correction. However, because TruSeq relies on long-range amplification and the reads are synthetically generated, the available read lengths are shorter than other approaches, and are prone to termination and biases in any region where the Illumina chemistry is biased, such as regions with high GC content or tandem repeats. Finally, obtaining sufficient coverage for *de novo* genome assembly can be expensive, often even greater than PacBio sequencing, since 900x to 1500x or more short read coverage may be required to assemble 30x coverage of synthetic long reads (Kuleshov *et al*., 2014; Lee *et al*., 2016).

Oxford Nanopore Technologies (ONT) has developed MinION which is the first available device for nanopore sequencing. These sequencing devices are with the size of a small cell phone and can be plugged into the USB of a laptop. The principle of the underlying technique is based on biological nanopores. Some toxins, such as the staphylococcal α-hemolysin protein, are able to form a pore within a phospholipid bilayer, thereby creating a small channel measuring a few nm in diameter (Feng *et al*., 2015). When placing the bilayer in a salt solution, electrodes can be used to form an ionic gradient. DNA (with net negative charge) can be forced by this gradient to pass the nanopore and each nucleotide passing the pore characteristically decreases the amplitude of the ion current, which can be detected by a sensor (Branton *et al*., 2008; Clarke *et al*., 2009).

The MinION sequencing device is equipped with 512 channels containing nanopores, each detecting ~ 10 bp per second. The sequencing is conducted by a method called strand sequencing. For sequencing library preparation, the ends of each DNA molecule are modified by ligating a hairpin adapter to one end and a motor protein to the other. The motor protein ratchets the DNA molecule through the nanopore, making it single-stranded. In the ideal case, one strand passes through the nanopore, followed by the hairpin-adaptor and the second strand of the molecule. Due to the length of the nanopore tunnel and the speed of the process, more than one nucleotide is always present in the pore at a time (Goodwin *et al*., 2015).

Accordingly, the signal of overlapping 5-mers is recorded, which means that the cloud-based base-calling software MinKNOW needs to distinguish 45 (1024) possible ionic current states for all possible 5-mers to generate the raw sequence. High error rate is reported for all reads produced by this technique so far, ranging from 25% to 40%. The output of the published studies ranged from 90 to 490 mbp per 48 hours, with average read lengths around 6 kbp and maximum read lengths of up to 150 kbp (Quick *et al*., 2014; Ashton *et al*., 2015; Goodwin *et al*., 2015; Laver *et al*., 2015). A benchmark study of the Min-ION Analysis and Reference Consortium reported that a typical experiment yields 20,000 2D-reads comprising 115 mbp with an error of ~12% (Ip *et al*., 2015).

Portability of the instrument's and low cost of the sequencing facility have empowered the Oxford Nanopore MinION to be used for studies in very remote locations, including studying Ebola outbreaks in the field in West Africa (Quick *et al*., 2016).

## 2.8 Plant Virus Management Options

Virus and viroid diseases of crops cause enormous losses on the quality as well as quantity of products (Sastry, 2013). Continuing disease in crop hosts requires a source or reservoir of virus. The development of effective disease control measures will require an understanding of the relative importance of these reservoirs in disease initiation. Since virus infection is systemic, no commercial viricides or chemical agents have yet been developed. However a number of indirect control measures for different crop plant virus/viroid pathosystems have been developed with an aim to avoid the pathogen or to minimize their impact on crop yields (Thresh, 2006).

An effective and applicable virus management strategy requires an accurate diagnosis and understanding the life and disease cycle of etiological agents. According to Sastry and Zitter (2014) there are two basic approaches to manage virus and viroid diseases of crop plants in general and maize in particular. The first is to decrease the sources of infection (reservoirs) and secondly to minimize the rate of spread using different methodologies. Similarly, Redinbaugh and Zambrano (2014) indicated the most common approaches for management of virus diseases in maize as (1) breaking virus-vector and virus-maize interactions by removing virus reservoirs; (2) breaking vector-maize interactions by reducing vector population on susceptible maize or deploying insect resistant crops; (3) breaking virus-maize interactions by using virus disease resistant crops; and (4) using clean seed and clean soil.

The particular choice of management strategies to be used depends on the nature of the particular virus and viroid, nevertheless the decision to use the control measures depends on the assessment of the economic risks involved in taking no action (Sastry and Zitter, 2014). Generally, the various management measures employed for virus and viroid disease management can be included in one of the following methods.

### 2.8.1 Host Resistance

The most economically viable and environmentally sustainable approach to control virus diseases in crops is to deploy virus-resistant cultivars and hybrids. This mainly requires identification and evaluation of resistant plants which then culminate by incorporating resistant alleles into agronomically desirable genetic backgrounds. Because the identified virus resistance in maize is primarily mono- or oligogenic, most of the methods employed for breeding virus resistance in maize include (i) screening germplasm collections for resistance; (ii) identification of markers linked to genes or QTLs conferring resistance; and (iii) introgression of the resistance into elite breeding lines (Redinbaugh and Zambrano, 2014).

Plants can fend off pathogens either by reducing or restricting pathogen growth (resistance) or by reducing or moderating pathogen effects (tolerance) (Roy and Kirchner, 2000; Boots, 2008). For maize, genes or QTL conferring moderate to strong resistance to most of the major viral diseases have been identified and the genetics of resistance have been defined (Redinbaugh and Pratt, 2008; Redinbaugh and Zambrano, 2014). Genetically engineered virus resistance, conferred by expression of viral sequences in plants, takes advantage of the plant's innate induction of RNAi against virus sequences known as virus-induced gene silencing (Lindbo and Falk, 2017). The recent development of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas for maize (Svitashev *et al.*, 2015) provides the possibility for modification of maize gene alleles to provide virus resistance that can be developed into lines and hybrids that do not have non-maize sequences in the genome. Alternatively, CRISPR-Cas for genome editing might be useful for engineering RNAi for virus resistance into maize genome (Zaidi *et al.*, 2016).

### 2.8.2 Vector Control

To date, the entire economically important virus diseases of maize are vector transmitted by arthropods (Mahuku *et al.*, 2015a). Thus, prohibiting the interaction of a vector with maize host by using treatments that decrease vector population is key step in maize virus disease control. Vector control through the application of chemical spray (insecticide) or transgenic approach might also be used. For example, in thrips RNAi of V-ATPase-B knocked down gene expression and protein levels increased female mortality, and decreased fecundity in *Frankliniella occidentalis*. Although more research is needed to understand the effectiveness of this approach under agronomic conditions, RNAi for thrips control might also be important for MLND management (Badillo-Vargas *et al.*, 2015).

Insecticide treatments directed at vector populations of persistently and semi-persistently transmitted viruses can reduce disease incidence (Antignus *et al.*, 2008). But insecticide treatments are not generally effective for non-persistently transmitted viruses that are transmitted within a few minutes of insect feeding (Jones, 2006). On the other hand adjusting planting dates and crop rotations have provided some disease control by limiting the access of crops to vectors (Fritts *et al*., 1999).

### 2.8.3 Clean Seed and Soil

Use of virus-free planting material with good genetic background and planting it on clean soil is a pre-requisite for good yield of a crop. After planting, there should be continuous follow up and subsequent precautionary measures. Moreover, continuous actions to restrict the spread of viral diseases using cultural, chemical or biological means need to be employed. Hence using virus-free seed and subsequent vector management are the most effective input in managing virus diseases, there by succeeding with higher yield (Sastry and Zitter, 2014).

### 2.8.4 Integrated Disease Management

The central idea for a virus disease to occur is that the three components including the virus, its vector, and a susceptible host have to come together in a suitable environment (Redinbaugh and Zambrano-Menzoda, 2014). Use of highly adapted, high-yielding maize hybrids and cultivars with durable, strong resistance to virus diseases would be ideal for disease control. However, this should be used in integration with cultural practices, insecticides and other improved agronomic practices for the best containment of maize virus diseases.

# Chapter 3

# 3. Survey for the identification and geographical distribution of viruses and virus diseases of maize (*Zea mays* L.) in Ethiopia

## Abstract

Maize is leading cerial crop in terms of production in Ethiopia. Introduction and rapid distribution of maize lethal necrosis disease (MLND) in Ethiopia has put significant burdon on maize production. A survey to identify the distribution of virus diseases of maize was made in major maize growing regions of Ethiopia. Four surveys were conducted between 2015 and 2017. A total of 284 maize fields have been surveyed, of which 846 leaf samples with virus like disease symptoms were collected from 191 fields. There were no symptoms indicating for virus presence in maize grown in 93 fields which were exempted from sampling, while in contrast up to 100% disease incidence was recorded in maize grown in the Benishangul-Gumuz, Oromia and South Nations, Nationalities and People (SNNP) regions. Symptoms varied in maize ranging from the most common mosaic and streak to severe leaf mottling and necrosis as well as stunting and pre-mature plant death. Double or triple antibody sandwich (DAS or TAS) enzyme-linked immuno-sorbent assay (ELISA) to test for eight common maize viruses such as *Maize chlorotic mottle virus* (MCMV), *Sugarcane mosaic virus* (SCMV), *Maize streak virus* (MSV), *Maize dwarf mosaic virus* (MDMV), *Maize mosaic virus* (MMV), *Maize stripe virus* (MSpV), *Wheat streak mosaic virus* (WSMV) and *Johnsongrass mosaic virus* (JGMV) confirmed the presence of MCMV, SCMV and MSV in the leaf samples. Single or mixed infections with MCMV and SCMV frequently occurred and triple infections of MCMV, SCMV and MSV were found in 1% of samples from SNNP region. Sequence analysis of the coat protein genes of randomly selected seropositive samples of the three viruses showed little variability within the studied isolates and those retrieved from the GenBank. Our results indicated MLND caused by MCMV and SCMV co-infection was the most important disease in SNNP and Oromia while maize streak disease was the predominant virus infecting maize in Benishangul-Gumuz.

## 3.1 Introduction

Maize (*Zea mays* L.) is Ethiopia's principal crop in terms of production and the second in area coverage next to the staple food crop tef [*Eragrostis tef* (Zucc.) Trotter]. A total of 8 million tons have been produced across 2.2 million hectares of land in the 2016 cropping season (CSA, 2017). Despite its national importance, however, the national average yield of maize in Ethiopia (3.4 tons/ha) is below the world's average yield (5.6 tons/ha) (FAOSTAT, 2014). Diseases of maize caused by viruses are serious threats to maize production in Ethiopia. Earlier studies of maize virus in Ethiopia revealed the presence of *Maize streak virus* (MSV; genus *Mastrevirus* in the family Geminiviridae), *Sugar cane mosaic virus* (SCMV), *Maize dwarf mosaic virus* (MDMV), *Johnson grass mosaic virus* (JGMV) all belonging to genus *Potyvirus* in the family *Potyviridae*, and *Maize mottle chlorotic stunt virus* (MMCSV) (Mesfin Tessera *et al*., 1991; Alemu Lencho *et al*., 1997).

However, the recently emerged maize lethal necrotic disease (MLND), which occurs when *Maize chlorotic mottle virus* (MCMV; the lone member of the genus *Machlomovirus* in the *Tombusviridae* family) infects maize along with SCMV, is the principal virus disease of maize in Ethiopia (Mahuku *et al*., 2015b; Mengistu Fantahun *et al*., 2017). Even though SCMV is the most common in Ethiopia and other countries in the Eastern region of Africa, different members of the *Potyviridae* family including *Wheat streak mosaic virus* (WSMV) in the genus *Tritimovirus* (Scheets, 1998), MDMV (Niblett and Claflin, 1978) or JGMV (Stewart *et al*., 2017) are known to result in MLND by forming synergy with MCMV. MLND is a great menace to maize based food security in other SSA countries as well (Mahuku *et al*., 2015a; Kiruwa *et al*., 2016).

MSV is common in Ethiopia in particular in low land areas and observed in most parts of Africa as well as several Indian islands as maize streak disease (MSD) (Shepherd *et al*., 2010). Another newly emerging maize virus, *Maize yellow mosaic virus* (MaYMV; genus *Polerovirus*; family *Luteoviridae*) was recently reported in different regions of Ethiopia (Demsachew Guadie *et al., 2018*), providing evidence for a steadily changing virus situation. All the above mentioned and other economically important viruses of maize are vectored by different arthropod species (Mahuku *et al*., 2015a).

In this report, we present results from comprehensive field surveys conducted over 3 years in five major maize growing regions of Ethiopia. Our goal was to determine, the viruses implicated in maize diseases in Ethiopia, map their geographical distribution and estimate their prevalence and importance. The results of the virus survey which were based on ELISA were further substantiated by molecular analysis of coat protein genes to evaluate variation and diversity.

## 3.2 Materials and Methods

### Field survey and collection of samples

Field surveys were conducted in Amhara, Benishangul-Gumuz, Oromia, Southern Nations, Nationalities and People (SNNP) and Tigray adiminstrative regions of Ethiopia in which maize is cultivated as one of a major crop. In these regions maize, in most cases, is cultivated in monoculture, as the major crop by small holder farmers (CSA, 2017). The surveys were made from August to September, 2015 (late main rain feed cropping season), in July 2016 (middle of main rain feed cropping season), in November 2016 (beginning of dry season, irrigation) and in March 2017 (middle of dry season) (Table 1). Most of the surveyed maize fields were at a vegetative growth stage ranging in size around knee height to early tasseling. Maize leaf samples from plants with conspicuous viral disease symptoms have been collected and dried over anhydrous calcium chloride prior to serological testing. The surveys were made along road-sides with approximately ten kilometer intervals wherever the crop was available. Passport data along with global positioning system (GPS) coordinates were registered for each field.

During sampling each field was divided in to two diagonals and five samples were collected, one around the center and the rest from the diagonals. In some fields with high disease incidence (≥80% symptomatic plants) and where unique symptoms were observed, up to 10 samples were collected to account for the different symptoms. Disease incidence was calculated as the percentage of plants showing the virus disease symptoms to the total number of plants observed in a field as described in James (1974). In 191 of 284 fields visited, 846 samples were collected. The remaining 93 fields, in which conspicuous viral symptoms were not observed, were exempted from sampling (Table 3.1). The collected maize leaf samples were dried over anhydrous calcium chloride prior to serological test.

**Table 3.1** Total number of fields surveyed and samples collected from all the surveys.

| No. | Survey Seasons[a] | Surveyed regions | Fields[b] |  | Samples[c] |
|---|---|---|---|---|---|
|  |  |  | With Symptom | No symptom |  |
| 1 | I | Amhara, Benishangul-Gumuz, Oromia and SNNP | 77 | 36 | 340 |
| 2 | II | Benishangul-Gumuz, Oromia, SNNP | 66 | 13 | 268 |
| 3 | III | Oromia and SNNP | 23 |  | 138 |
| 4 | IV | Amhara and Tigray | 25 | 44 | 100 |
| **Total** |  |  | **191** | **93** | **846** |

[a] survey seasons = maize field survey seasons: main cropping seasons with long-rain, August to September 2015 (I) and July 2016 (II); off cropping seasons with short or no rain but irrigation, November 2016 (III) and March 2017 (IV).

[b] total number of maize fields with different viral symptoms and without any observable viral symptom surveyed in all survey seasons across different regions of Ethiopia.

[c] total number of samples collected from maize fields with single or a combination of different viral disease symptoms.

### ELISA

All the collected samples were subjected to ELISA against eight common maize viruses. The detection antibodies for MCMV (AS-1087), SCMV (AS-0166), (WSMV) (AS-0544), JGMV (AS-0197) and MDMV (AS-0519-0662/2) and their respective positive controls were provided from the DSMZ-Plant Virus Collection, Germany. ELISA tests for detection of MSV (Cat No. 18800), *Maize mosaic virus* (MMV; genus *Nucleorhabdoviridae*; family *Rhabdoviridae*) (18900) and *Maize stripe virus* (MSpV; genus *Tenuivirus*; family *Phenuiviridae*) (17300) with their respective positive controls were purchased from AGDIA (USA).

Since monoclonal antibodies which increase specificity of testing were obtained from the DSMZ, MDMV was tested with triple antibody sandwich (TAS) ELISA and all other test formats were double antibody sandwich (DAS) ELISA. Tests were conducted following the standard protocols at the DSMZ-plant virus collection. Absorbance values to measure substrate conversion were determined in an ELISA plate reader at $E_{405}$ nm. Values greater than three times the mean value recorded for the healthy control were considered as ELISA positive for the tested viruses.

In DAS ELISA pecific antibodies (γ globulin IgG) of the respective viruses were diluted with coating buffer as recommended in the standard protocol, for example, 1 μl of IgG in 1 ml of coating buffer with a recommended dilution of 1:1000 or 1 μl of IgG in 0.5 ml of coating buffer with a recommended dilution of 1:500. Then 200 μl of the diluted IgG was dispensed to each well of the greiner bio-one polystyrene microtiter ELSA plate. Each plate was sealed with thin polyethylene plastic cover and incubated at 37 °C for 2 hours. After incubation the plates were washed three times with PBS-Tween (Appendex I) by soaking for few minutes in between washing steps. After the final washing step the plates were allowed to dry by tapping upside down on tissue paper.

Then 200 μl aliquots of the test samples which were extracted from dried leaf samples with extraction buffer in 0.5:20 (W/V) were added in duplicate wells of the coated plates. The last three duplicate wells of each plate were loaded with negative, positive and buffer controls, respectively. All the plates were covered with thin polyethylene plastic and incubated over night at 4 °C. The next day plates were washed 3x as the previous washing step and allowed to dry. This was followed by addition of 200 μl of specific enzyme conjugated antibodies (IgG-AP) of the respective viruses diluted in conjugate buffer following the recommended dilution factors. The plates were sealed and incubated at 37 °C for 2 hours followed by washing three times with PBST and drying.

Finally, 200 µl aliquots of freshly prepared substrate (1 mg/ml para- nitrophenyl-phosphate, disodium salt (PNPP) in substrate buffer) was added to each well, sealed and incubated at 37 °C for 30-60 min, or as long as necessary to obtain clear reactions. Absorbance values were determined in an ELISA plate reader at $E_{405}$ nm.

For TAS-ELISA, after washing and drying of the coated plates 200 µl of 2 % (W/V) skimmed milk in PBST was added to each well as a blocking agent and the plates were covered and incubated at 37 °C for 30 min. The blocking agent was removed and the plates were immediately dried by tapping without washing. Then 200 µl aliquots of the test samples were added in duplicate wells, covered and incubated overnight at 4 °C. The plates were washed, dried and 200 µl of specific monoclonal antibody (mAb) diluted with the recommended dilution of the conjugate buffer were added in duplicate wells. The plates were covered and incubated at 37 °C for 2 h followed by washing and drying.

Two hundred µl of RAM-AP (rabbit Ig anti-mouse Ig alkaline phosphatase) diluted with recommended dilution factor in conjugate buffer were added to each well of the plate, covered and incubated at 37 °C only for 1 h. Then 200 µl aliquots of the test samples were added in duplicate wells, covered and incubated overnight at 4 °C. This was followed by the procedures for DAS-ELISA to identify positive and negative samples.

### Nucleic acid extraction, PCR, Cloning and Sequencing

A further confirmation of ELISA positive samples by PCR followed by sequencing was done with selected samples to evaluate the diversity in the coat protein genes of MCMV, SCMV and MSV. Six ELISA positive samples for each species were randomly selected and tested by PCR using specific primers amplifying full length coat protein genes. For MCMV and SCMV, total RNA was extracted from ~20mg dried maize leaves using Epoch Life Science GenCatch Plant RNA purification Kit (Cat No.: 17-60250) following the manufacturer's instructions. For MSV, total DNA was extracted from the dried leaf samples using Qiagen DNeasy Plant Mini Kit (Cat No.: 69104) according to the manufacturer's instructions.

For MCMV and SCMV coat protein gene cDNA was synthesized prior to PCR at 45 °C for 50 min using the respective reverse primers (R). Followed by PCR using the primers for MCMV (F 5' -GTCCTGGCCTCAGTGGTTAAGG-3′, R 5'-TCTCCAGTCATGGTCATCACGC-3′) and for SCMV (F 5′-GCGRTGGCTTYTSGAAATGCAACC-3′, R 5′-AGAAGACACTGGGTCCAACCCTG-3′). For MSV PCR was done using F 5'-TGAAGGCTCGRCAAGGCAGAT-3′ and R 5′-TTCRATGTTYTGCCCGCCGAG-3′ primers.

The coat protein genes were amplified using Phusion Flash High-Fidelity Master Mix (Cat. No.: F548, Thermo Fisher Scientific). The reaction was performed in a 20 µl total reaction volume containing 10 µl 2x Phusion Flash buffer, 2 µl cDNA/DNA, 0.5 µl from each of the forward and reverse primers and 7 µl nuclease free water. For MCMV and SCMV the PCR programming was adjusted at 98 °C pre-heating for 30 s followed by 35 cycles of 98 °C for 10 s, 59 °C for 20 s, 72 °C for 40 s followed by final extension for 10 minutes at 72 °C. For MSV all the PCR program was the same except the annealing temperature was raised to 61 °C.

The PCR products were separated using 1% (w/v) agarose gel immersed in 1x Tris Acetate-EDTA (TAE) electrophoresis buffer at 120 V for 45 minutes and stained with Midori Green (Nippon Genetics), purified from agarose gel using Nucleospin gel and PCR clean up kit (Macherey-Nagel), Germany and ligated to pJET1.2/blunt vector (Cat. No.: K1232, Thermo Fisher Scientific).

The ligation reaction was performed in a 10 µl total reaction volume containing 5 µl of 2x reaction buffer, 0.5 µl pJET vector, 0.5 µl ligase and 4 µl of the respective coat protein gene fragments and incubated over night at 4 °C. The ligated products were subsequently transformed to electrocompitent DH5α *E. coli* host cells. For transformation ~ 40 µl of host cells were collected from -80 °C freezer and aliquated on ice for about 5 minutes and thoroughly mixed with 2 µl of the ligated products by pipetting in and out.

The whole mix was pipetted out in to an ice cold cuvette and immediately electropotrated at 2.5 kV for 5 microseconds. The cells were immediately rescued with 1 mL Super Optimal broth with Catabolite repression (SOC) medium (Sigma-Aldrich), thoroughly mixed and transferred to a sterile pre-labeled eppendorf tubes. To recover the transmormants, the tubes were incubated at 37 °C for an hour while shaking at 1,800 rpm. Hundred µl of the incubated cells were plated over agar plates containing 100 mg/ml ampicillin in two replications and allowed to grow overnight at 37 °C. Overnight grown colonies (one colony from each plate) were inoculated in a test tube containing 2 ml LB liquid medium mixed with ampicillin in 1:1000 ratio. Multiplied plasmids were extracted from overnight cultures using Nucleospin plasmid extraction kit (Macherey-Nagel), Germany. One clone for each fragment was sequenced from both orientations using the sequencing service at Helmholtz Center for Infection Research, Genomanalytik, GMAK, Germany.

### Sequence analysis

Forward and reverse sequence reads of each isolate were individually assembled and edited by Geneious v 10.2.3 (Biomatters LTD, NZ) excluding the primer sequences. For phylogenetic assessment, the sequences were trimmed to the coat protein coding gene and compared with sequences of related viruses available at NCBI GenBank. For SCMV the sequences were translated to amino acid sequences and conceptually trimmed at the NIb↓CP cleavage site at the N-terminus of the coat protein and the gene ORF sequences were inferred. Multiple sequence alignments were carried out using Clustal Omega (Sievers *et al*., 2011). The Phylogenetic trees were generated with MEGA 7.0.1 software using Neighbour-Joining (NJ) method (Kumar *et al*., 2016) and a Bootstrap value of 1000 replicates.

## 3.3 Results

### Field survey

During virus surveys between 2015 and 2017 maize fields across five regions of Ethiopia were visited. Symptoms conspicuous for viral disease were found on leaves and those consisted of bright chlorosis, bleaching and stunting symptoms typical for MLND, streak symptoms, mosaic and chlorosis on the entire leaf lamella, broken or longitudinal chlorotic streaks along the leaf veins, wilting and drying of leaf margin, necrosis and mottling on the entire plant, “dead-heart” symptoms, shortened male inflorescence with few spikes, stunted growth, dwarfing, malformed or lacking ear sets and rotting or drying of cobs. In addition, unusual symptoms suspected of virus origin were found in the central and northern parts of Ethiopia (Fig. 3.1). Those included whitish to yellowish stripes, swollen veins, fine streaks and stunted growth.

### ELISA

ELISA for virus detection revealed the occurrence of only three viruses MCMV, SCMV and MSV in the surveyed areas. From all 846 samples, 171 (20%) were infected with MCMV, 50 (6%) were infected with SCMV and 97 (12%) were infected with MSV in single infections. MCMV + SCMV mixed infections were found in 217 (26%) samples from maize showing severe MLND symptoms. Two samples were positive for MCMV, SCMV and MSV in triple infection (Table 3.2).

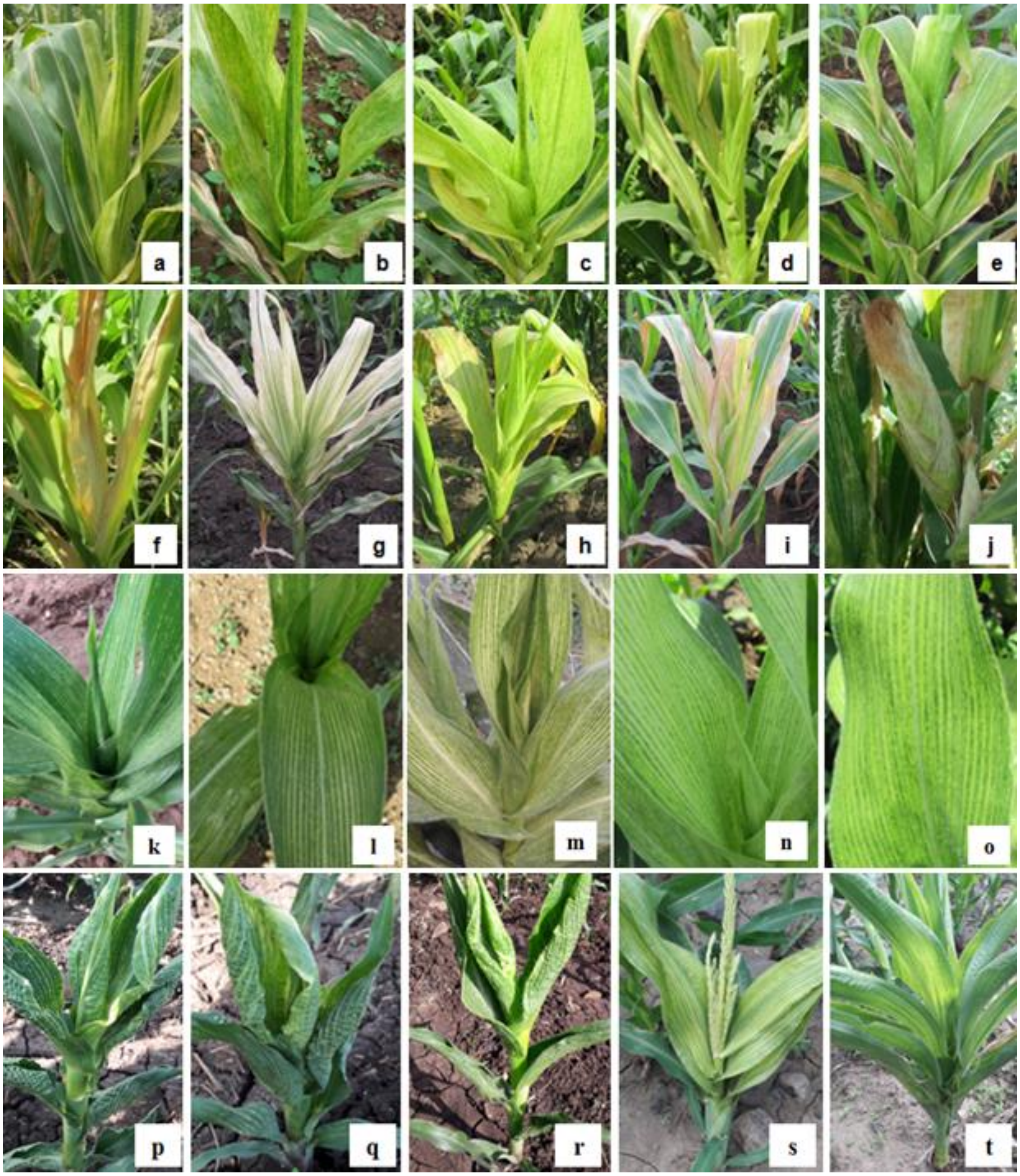


**Figure 3.1** Symptomatic maize seedlings showing different symptoms of MCMV single infection (a-e); MCMV and SCMV co-infection or MLND (f-j); MSV single infection (k-o) and unusual virus disease like symptoms (p-t) observed during the field survey from different regions of Ethiopia.

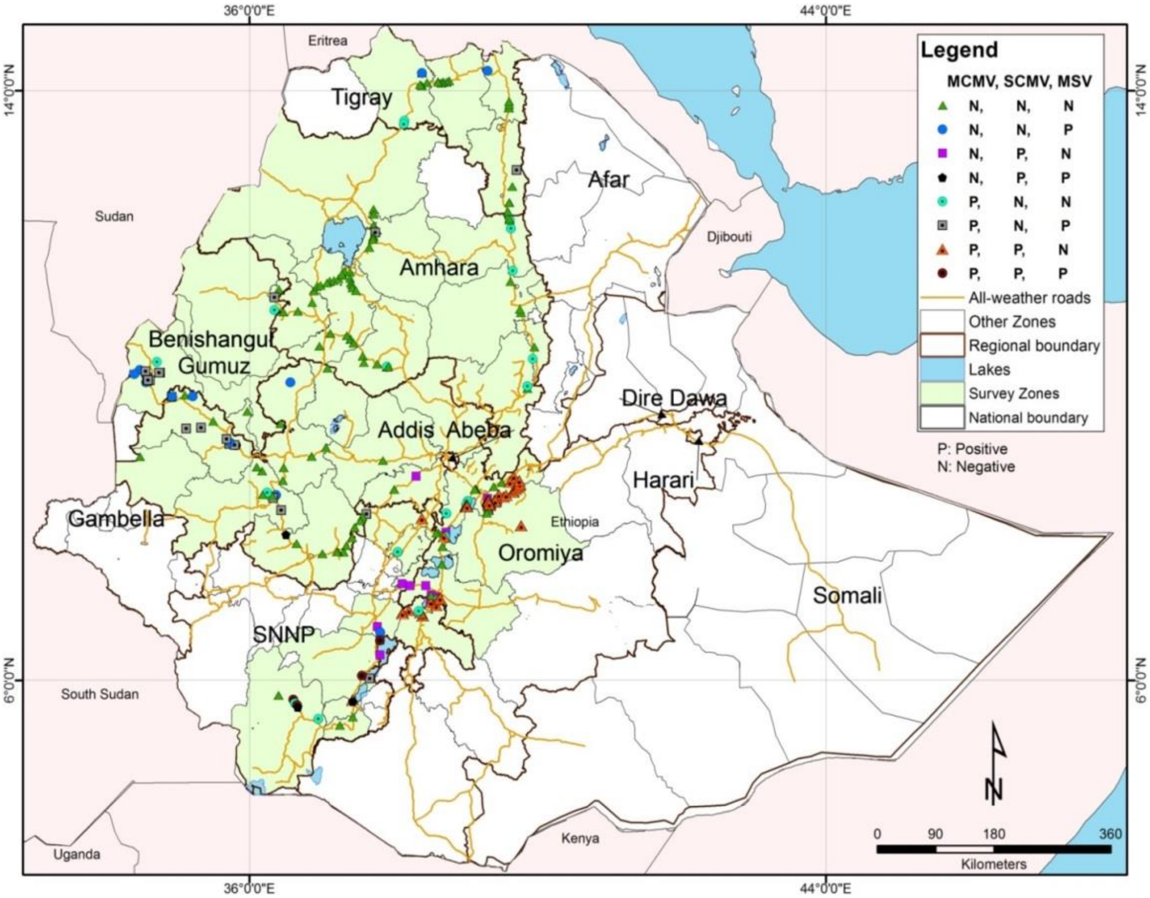


**Figure 3.2** Map of Ethiopia showing geographical distribution of the three viruses in single or mixed infections in the surveyed regions.

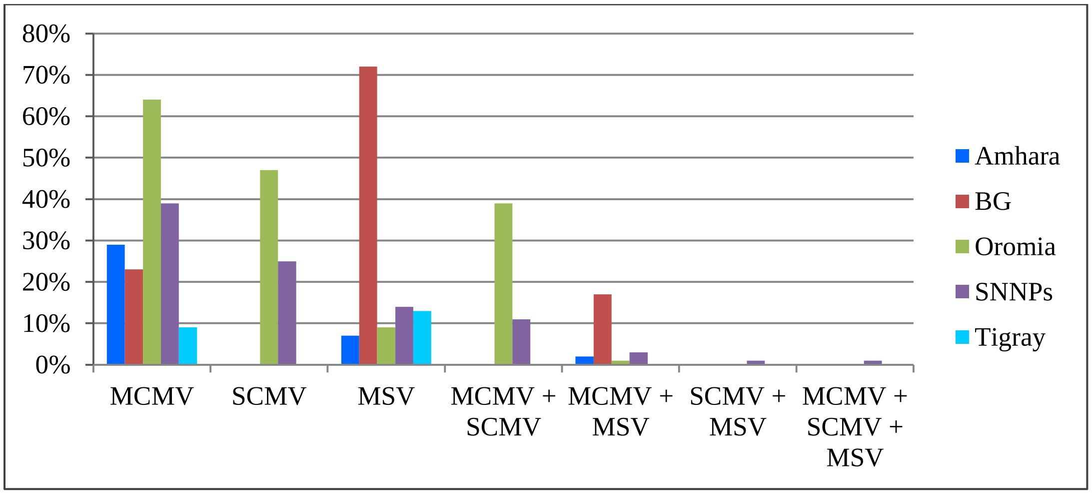


**Figure 3.3** Regional distributions of MCMV, SCMV and MSV in single or different combinations of mixed infections.

### Distribution, disease incidence and prevalence

During surveys, based on symptom monitoring the highest disease incidence (up to 100%) as a result of MLND and/or streak like symptoms were observed from Benishangul-Gumuz, Oromia and SNNP regions which are characterized by low to mid altitude ranges. The incidence slightly decreases towards the highland areas in Amhara and Tigray regions. Based on ELISA, MCMV, SCMV and MSV were found in all of the five surveyed regions except for SCMV which was not present in Amhara, Benishangul-Gumuz and Tigray regions (Table 3.2; Fig. 3.3).

Higher disease incidence was found in low to mid altitudes of different regions. For example, 46% fields in altitude ranges from 1168 to 1939 masl, 50% in ranges from 1139 to 1790, 81% in ranges from 1333 to 1597, 21% in ranges from 1113 to 1640 and 23% in ranges from 1297 to 2199 were identified with at least 50% disease incidence in Oromia, SNNP, Benishangul-Gumuz, Amhara and Tigray regions, respectively.

Up to 100% diseased maize was found in Benishangul-Gumuz region which is characterized by lower elevations than the other surveyed regions. The higher disease prevalence in lower altitudes of SNNP (83%) and Oromia (70%) declines in higher altitudes to 19% (in Tigray) and 18% (in Amhara) regions (Table 3.3).

**Table 3.2** Comparison showing incidence of MCMV, SCMV or MSV and mixed infections of samples collected in all survey seasons.

| Season[f] | Region[g] | Altitude Range(masl) | Incidence Range (%) | Samples[h] | ELISA positive samples (%)[e] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | MCMV | SCMV | MSV | MCMV +SCMV | MCMV +MSV | SCMV + MSV | MCMV + SCMV + MSV |
| 2015(I) | Amhara | 1033-2475 | 10-70 | 31 | 1(3) | | 2(6) | | 2(6) | | |
| | Benshangul-Gumuz | 1333-1597 | 40-100 | 55 | 2(4) | | 32(58) | | 11(20) | | |
| | Oromia | 1344-2416 | 5-100 | 223 | 28(13) | 1(0.5) | 28(13) | 135(61) | 6(3) | | |
| | SNNP | 1763-2166 | 60-70 | 31 | 17(55) | 1(3) | | 7(23) | | | |
| | **Total** | | | **340** | **48(14)** | **2(0.6)** | **62(18)** | **142(42)** | **19(6)** | | |
| 2016(II) | Benshangul-Gumuz | 1537-1539 | 5-10 | 9 | 2(22) | | 3(33) | | | | |
| | Oromia | 1143-2226 | Trace-75 | 152 | 28(18) | 28(18) | 8(5) | 17(11) | 2(1) | 1(0.7) | |
| | SNNP | 1139-1881 | Trace-100 | 107 | 15(14) | 19(18) | 17(16) | 3(3) | 4(4) | 2(2) | 2(2) |
| | **Total** | | | **268** | **45(17)** | **47(18)** | **28(10)** | **20(8)** | **6(2)** | **3(1)** | **2(0.8)** |
| 2016(III) | Oromia | 1147-1758 | Trace-60 | 115 | 46(40) | 1(1) | | 50(44) | | | |
| | SNNP | 1462-2017 | Trace-50 | 23 | 8(35) | | | 5((22) | | | |
| | **Total** | | | **138** | **54(39)** | **1(0.7)** | | **55(40)** | | | |
| 2017 (IV) | Amhara | 1375-2395 | Trace-50 | 56 | 20(36) | | | | 2(4) | | |
| | Tigray | 1296-2161 | 1-60 | 44 | 4(9) | | 7(16) | | | | |
| | **Total** | | | **100** | **24(24)** | | **7(7)** | | **2(2)** | | |
| | ***Total*** | | | ***846*** | ***171(20)*** | ***50(6)*** | ***97(12)*** | ***217(26)*** | ***27(3)*** | ***3(0.4)*** | ***2(0.2)*** |

[e] ELISA positive samples (%) = the number of ELISA positive samples and their respective percentage in parenthesis for single or mixed infections

[f] season = survey seasons, from August to September 2015 (I); in July 2016 (II); in November 2016 (III); in March 2017 (IV)

[g] region = major maize growing regions of Ethiopia in which the surveys had been made

[h] samples = total number of samples collected from different regions of Ethiopia in different survey seasons

**Table 3.3** Regional and national prevalence of MLN and streak diseases of maize through ELISA test in major maize growing regions of Ethiopia.

| Region | Total No. of fields surveyed[a] | Altitude range of surveyed fields | No. of fields with MCMV/SCMV or MLND[b] | No. of fields with MSV[c] | No. of fields with at least one of the viruses[d] | Prevalence of all viruses[d/a] |
|---|---|---|---|---|---|---|
| Amhara | 66 | 1033-2475 | 10 | 2 | 12 | 18% |
| Benishangul-Gumuz | 16 | 1333-1597 | 10 | 14 | 16 | 100% |
| Oromia | 131 | 1143-2426 | 82 | 16 | 91 | 70% |
| SNNP | 40 | 1139-2166 | 32 | 9 | 33 | 83% |
| Tigray | 31 | 1297-2199 | 3 | 4 | 6 | 19% |
| **National Prevalence** | **284** | | **137** | **45** | **158** | **56%** |

[a] total No. of fields surveyed = total number of maize fields surveyed with or without conspicuous virus like disease symptoms

[b] No. of fields with MCMV/SCMV or MLND = total number of maize fields with either MCMV or SCMV single infection or co-infection to result in MLND

[c] No. of fields with MSV = total number of maize fields with streak disease symptoms

[d] No. of fields with at least one of the viruses = total number of maize fields with at least single infection of MCMV, SCMV or MSV

### Disease incidence variation in different survey seasons

The virus composition of samples collected during different cropping seasons also varied. A high incidence of MSV (58%) was found in those samples surveyed from Benishangul-Gumuz region during August to September, 2015 (late main rain feed season). In contrast, none of the samples collected in November 2016 (beginning of dry season, irrigation) were positive for MSV. Similarly, a high incidence of MCMV (55%) was found in maize samples from SNNP region during August to September, 2015 and for SCMV (18%) in Oromia and SNNP in samples collected during July, 2016 (main rain feed season). A high incidence of MLND (61%) was found in the Oromia region in samples collected during August to September, 2015.

### PCR and Sequencing

Expected sizes of the respective coat protein genes of MCMV, MSV and SCMV clearly appeared on 1 % agarose gel (Fig. 3.4) and obtained by purification. The cloned products of respective coat protein genes (Fig. 3.5) were sequenced and registered on the NCBI GenBank for MCMV (Accessions: MH286953-MH286958) (Fig. 3.6A), SCMV (MH286965-MH286970) (Fig. 3.6B) and MSV (MH286959-MH286964) (Fig. 3.6C) were provided.

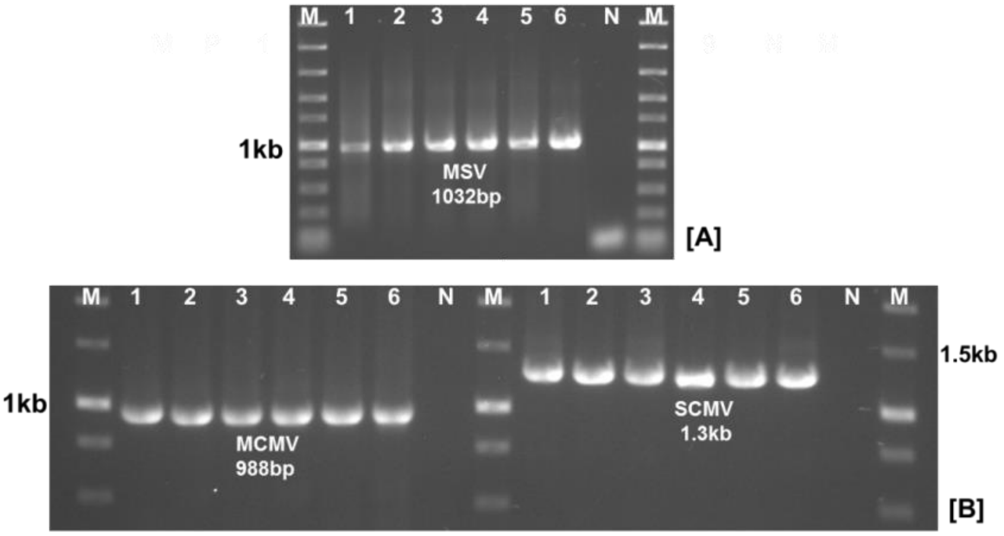


**Figure 3.4** PCR (A) and RT-PCR (B) products of coat protein genes of MSV (A); MCMV (B on the left) and SCMV (B on the right); N stands for negative control. Fast DNA ladder (NEB) was used as size marker.

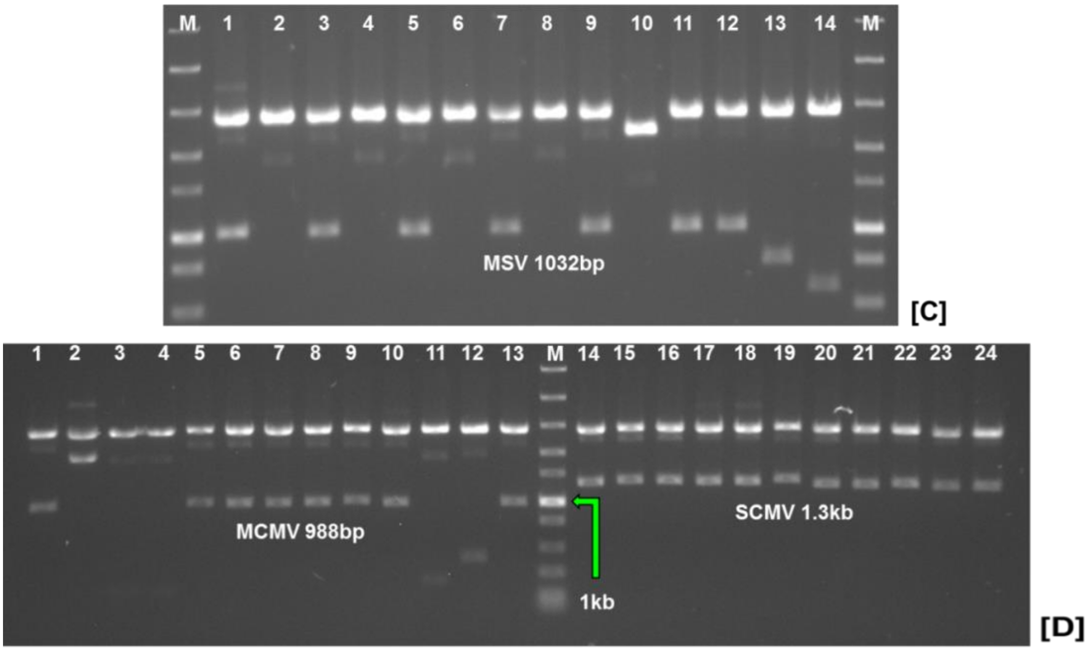


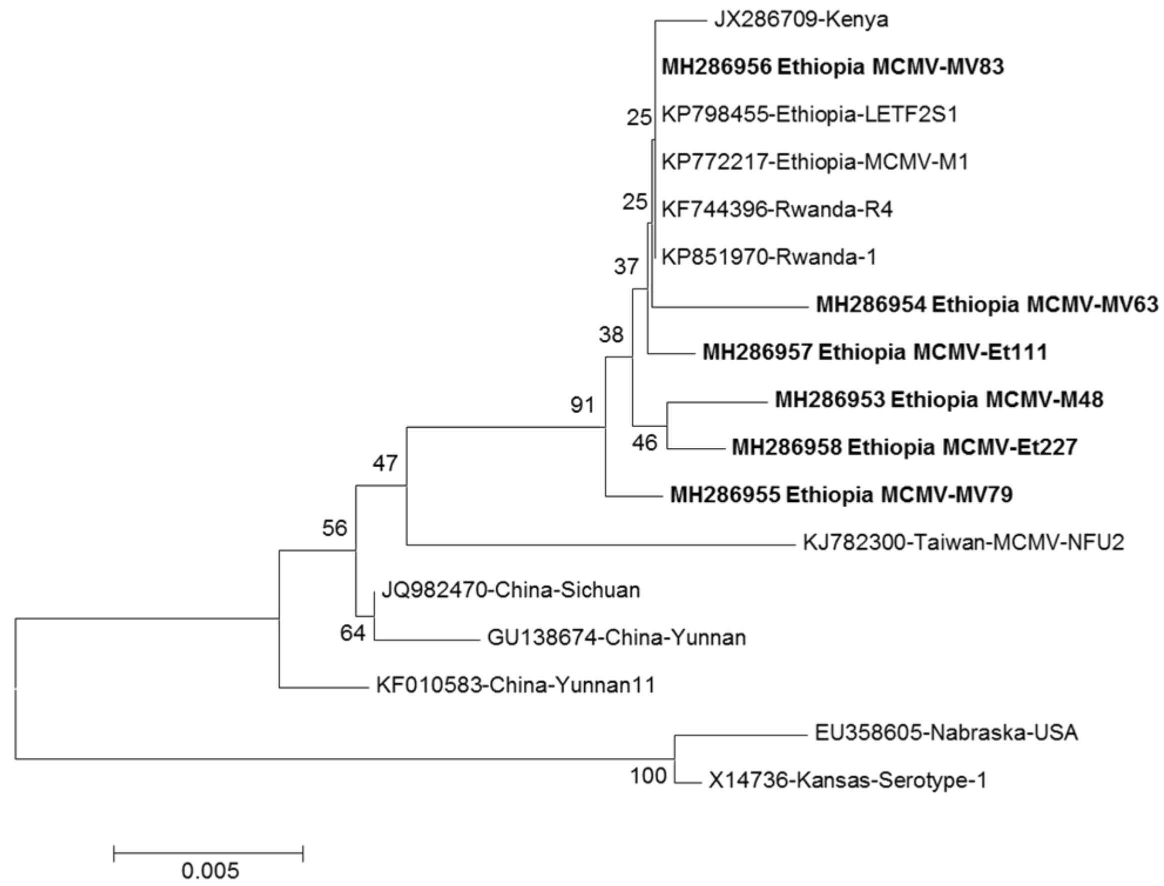
**Figure 3.5** Electrophoresis of the cloned products of MSV [C]; MCMV (left) and SCMV (right) [D] coat protein genes in pJET vector after restriction digestion with *Bgl* II restriction enzyme. Fast DNA ladder was used as size marker.

### Coat protein gene sequence variation

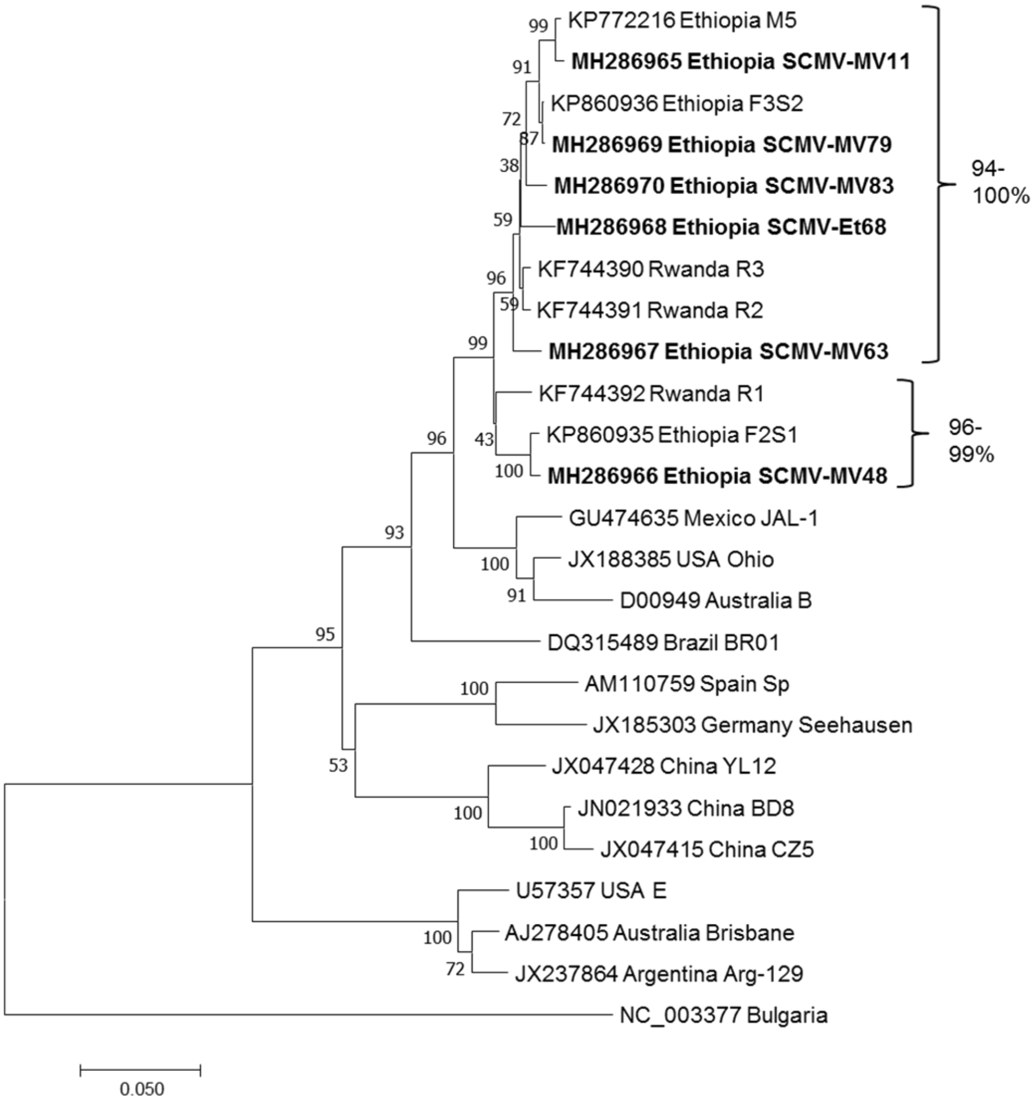
After trimming the 3’ and 5’ ends of coat protein gene nucleotide sequences, phylogenetic trees were constructed for coat protein gene sequences of MCMV (711 bp), MSV (735 bp) and SCMV (987 bp) virus isolates, respectively.

The six MCMV isolates, shared 99% to 100% nucleotide identity among themselves and with the East African isolates including two Ethiopian isolates (KP798455 and KP772217) available at GenBank. All the isolates from Ethiopia and other East African countries including isolates of this study shared 98% and 96% with the Asian and American Isolates, respectively (Fig. 3.6A).

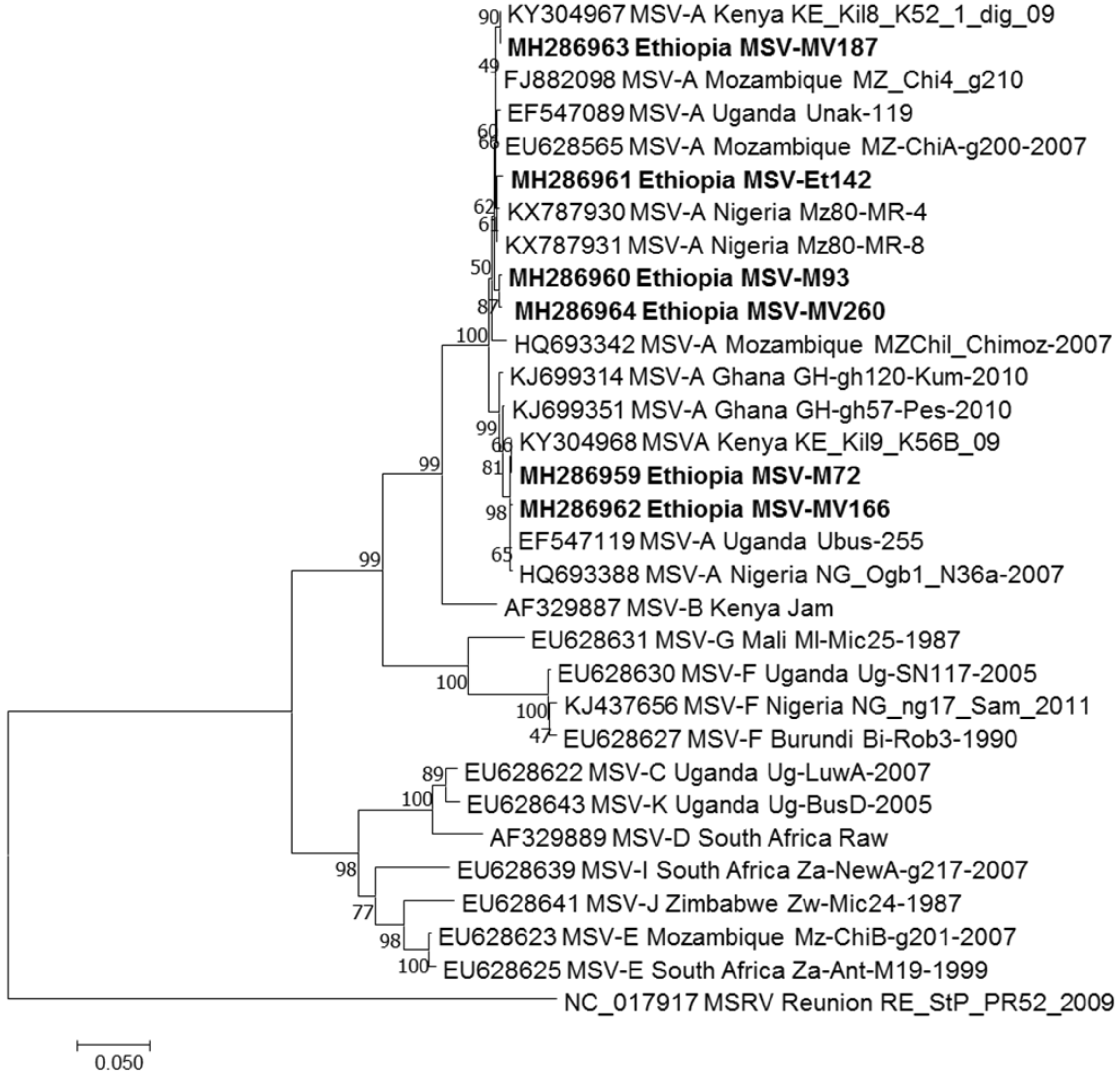
The SCMV isolates identified in this study shared 94 to 98% nucleotide sequence identity and were from 81 to 100% identical to sequences retrieved from GenBank (Fig. 3.6B). One of the SCMV isolates from the Oromia region has 945 bp due to 42 bp internal deletion near the 5′ end confirming the abnormality found in SCMV sequence from an earlier study from Ethiopia (KP860935). One of our isolates (MH286970) shared 100% nucleotide identity with previously reported isolate from Ethiopia (KP860936). All the 18 isolates retrieved from the GenBank and used in the phylogenetic relationship analysis have three categories of coat protein gene that is, nine isolates have 987 bp, five isolates have 942 bp and four isolates have 945 bp including the last stop codon. Whereas those of the MSV isolates used in this study shared 97% to 100% nucleotide sequence identity as well as with those isolates retrieved from the GenBank (Fig. 3.6C).


JX286709-Kenya
MH286956 Ethiopia MCMV-MV83
KP798455-Ethiopia-LETF2S1
KP772217-Ethiopia-MCMV-M1
KF744396-Rwanda-R4
KP851970-Rwanda-1
MH286954 Ethiopia MCMV-MV63
MH286957 Ethiopia MCMV-Et111
MH286953 Ethiopia MCMV-M48
MH286958 Ethiopia MCMV-Et227
MH286955 Ethiopia MCMV-MV79
KJ782300-Taiwan-MCMV-NFU2
JQ982470-China-Sichuan
GU138674-China-Yunnan
KF010583-China-Yunnan11
EU358605-Nabraska-USA
X14736-Kansas-Serotype-1
25
25
37
38
91
46
47
56
64
100
0.005

[A]


99
KP772216 Ethiopia M5
MH286965 Ethiopia SCMV-MV11
91
KP860936 Ethiopia F3S2
72
87
MH286969 Ethiopia SCMV-MV79
38
MH286970 Ethiopia SCMV-MV83
59
MH286968 Ethiopia SCMV-Et68
KF744390 Rwanda R3
96
59
KF744391 Rwanda R2
99
MH286967 Ethiopia SCMV-MV63
94-100%
KF744392 Rwanda R1
96
43
KP860935 Ethiopia F2S1
100
MH286966 Ethiopia SCMV-MV48
96-99%
GU474635 Mexico JAL-1
93
100
JX188385 USA Ohio
91
D00949 Australia B
95
DQ315489 Brazil BR01
100
AM110759 Spain Sp
JX185303 Germany Seehausen
53
JX047428 China YL12
100
JN021933 China BD8
100
JX047415 China CZ5
U57357 USA E
100
AJ278405 Australia Brisbane
72
JX237864 Argentina Arg-129
NC_003377 Bulgaria
0.050

[B]



[C]

**Figure 3.6** Phylogenetic tree reconstructed based on coat protein genes of MCMV, SCMV and MSV isolates determined in these study and those retrieved from the GenBank: MCMV (A), SCMV (B) and MSV (C). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Since it is the only species in the genus *Machlomovirus* no out group species was used in the phylogenetic tree construction of MCMV isolates. Maize dwarf mosaic virus (MDMV) (NC_003377) was used to bootstrap the phylogenetic tree of SCMV isolates. For MSV Maize streak Reunion virus (MSRV) (NC_017917) was used as an out group species and at least one isolate from each strains of MSV (A-K) were included for comparison. Isolates determined in this study are indicated in bold with the accession numbers provided from GenBank. GenBank accession numbers, country of origin and isolate names are shown for each isolate.

### 3.4 Discussion

Three virus species MCMV, SCMV and MSV were found almost in all of the main maize growing regions of Ethiopia. The higher incidences of MCMV and MLND were observed in the lower altitudes of Oromia and SNNP regions (between 1143 to 1881 masl). Maize treak disease incidences was higher in Benishangul-Gumuz and SNNP regions in areas of 547 to 1637 masl which can be attributed to warmer environmental conditions favoring the increase of vector population for virus transmission as well as contributing to severe disease symptoms (Redinbaugh and Zambrano, 2014).

MCMV was found in all surveyed regions of Ethiopia. The ecological niche modeling developed by Isabirye and Rwomushana (2016) using the Genetic Algorism for Rule-set prediction (GARP) ranked Ethiopia as the largest suitable habitat for MCMV, covering a total area of around 662, 974km$^2$ and a proportional potential yield loss of 60%. From our result as MCMV is distributed in the maize regions of Ethiopia, disease severity and yield loss is considerable because of the SCMV synergism (Alemu Lencho *et al*., 1997; Mahuku *et al*., 2015a; Mengistu Fantahun *et al*., 2017) resulting in MLND (Wangai *et al*., 2012a).

In our study, most of the samples which were positive for SCMV were found co-infected with MCMV confirming survey results made in other countries of Eastern Africa (Mahuku *et al*., 2015b). A high prevalence of maize virus disease in general was found in low to mid altitude areas of Benishangul-Gumuz, SNNP and Oromia regions.

The survey results of different cropping seasons in three consecutive growing years, showed that disease incidence was higher in August to September, 2015 than in July, 2016. This may be due to the fact that the vector populations increased significantly early in the season to result in a high disease incidence onwards. On the other hand, the disease incidence was higher in the off season (little to no rain or irrigation) in November, 2016 than the main rain feed season, July 2016. This again may be due to the warmer environmental conditions during the off-season which favors vector populations that transmit the disease over wider geography.

Comparisons of the coat protein coding genes of MCMV showed relatively little variability. This may be explained by the only recent introduction of MCMV to the Eastern region of Africa, however, MCMV is a virus with generally low diversity. MSV is an African virus and widely distributed on the continent including Ethiopia (Thottappilly *et al.*, 1993; Rybicki, 2015). This may explain the 3% nucleotide variation in the coat protein genes of the six virus isolates examined. The SCMV isolates were comparatively divergent than the MCMV and MSV isolates. Our results showed that there are at least two groups of SCMV isolates from Ethiopia with two categories of coat protein coding regions in size.

Our survey results indicated that MLND is established in all major maize production areas of Ethiopia and presents a serious constraint to maize production in central and southern regions, further spreading to high altitudes. Even though, it was not included in our study, future surveys should include possible alternative hosts harboring MCMV, SCMV and MSV in Ethiopia. There are different insect vectors such as corn thrips, corn root worms, corn flea beetles and cereal leaf beetles transmitting MCMV

(Nyvall, 1999). But there is no clear evidence whether these vectors or new species are facilitating the transmission of MCMV in Ethiopia. There are also controversies on the transmission rates of MCMV with infected seeds. Moreover, the transmission potential of contaminated soil from MCMV infected fields was proven to be very high (Mahuku *et al*., 2015b). Therefore, urgency is accentuated to increase efforts at devising effective disease control by considering maize lines resistant against the viruses and their virus complexes as well as strategies to control the disease using phytosanitation and integrated crop management options.

In conclusion, although symptoms conspicuous for virus presence were observed in a good number (24%) of maize plants, ELISA failed to detect any of the eight virus common to maize. In those samples with unique symptoms, it was possible that viruses which were not covered with ELISA tests could present. For some samples we employed PCR screening and Next Generation Sequencing (NGS) and confirmed the presence of a recently emerged maize virus, MaYMV (Demsachew Guadie *et al*., 2018).

Further quantitative studies are necessary to determine a putative wider distribution of this virus and to assess the importance of MaYMV to maize diseases in Ethiopia. The absence of WSMV, MDMV, JGMV, MMV and MSpV included in our test may be an indicative that these viruses are either do not exist in the country, existed in low concentration that cannot be detected by ELISA or rarely exist that they escaped sampling. These viruses can therefore be considered of little or no economic importance on maize yield loss in Ethiopia.

# Chapter 4

# 4. Distribution and Molecular analysis of maize (*Zea mays* L.)-infecting mastreviruses in Ethiopia

## Abstract

Maize (*Zea mays* L.) is host for more than 50 virus species worldwide with *Maize streak virus* (MSV) (genus *Mastrevirus*) causing significant yield losses in Africa. A survey for viruses infecting maize was conducted in major growing regions of Ethiopia. To test for DNA viruses, in particular mastreviruses, rolling circle amplification (RCA) was performed for analysis of virus composition in assayed samples. Following the analysis of the entire virus genomes, three genetic groups, each representing distinct virus species, were identified. The first group was almost identical with the A-strain of MSV. The next sequence-cluster shared 96-98% identity with isolates of *Maize streak reunion virus* (MSRV) confirming the presence of this virus also in continental East Africa. Sequence analysis of additional virus genomes (each 2846 nt) in length revealed only a limited 70-71% nt identity with MSRV isolates and an even lower identity (< 64%) with sequences of mastreviruses described elsewhere. Our analysis suggests a novel virus species, which is tentatively named maize streak dwarfing virus (MSDV). The pairwise comparison of capsid protein (CP) and replication-associated protein (Rep) of the novel species revealed a limited identity of 63% and 68% with the respective protein sequences of MSRV. The incidence of the virus species in the maize regions of Ethiopia was studied across 89 samples collected during four growing seasons. PCR analysis with general and specific mastrevirus primers showed that MSV is the most incident virus (39.3%) followed by MSRV (14.6%) and MSDV (12.4%). Identification of three different mastrevirus species in a confined geographical location on the same host, maize, is unprecedented, and suggests that Ethiopia may be one of the potential hot spots for diversity of maize infecting mastreviruses.

## 4.1 Introduction

Maize (*Zea mays* L.) which is the major staple food crop in Sub-Saharan African countries is leading cereal crop in terms of production in Ethiopia (FAOSTAT, 2017). Its production, however, is constrained by different biotic and abiotic factors. Among the biotic factors, viruses contribute significant yield loss and more than 50 species of virus have been identified to infect and cause disease on maize (Redinbaugh and Zambrano, 2014). Among these, *Maize streak virus* (MSV) belonging to the genus *Mastrevirus* in the *Geminiviridae* family is one of the top ten economically important plant viruses (Rybicki, 2015).

The two plant virus families *Geminiviridae* and *Nanoviridae* have a circular single-stranded (ss) DNA genome which replicates in the nuclei of their host plants via a mechanism called Rolling Circle Replication (RCR). Based on their genome organization, insect vector involved in transmission, and host range; viruses in the *Geminiviridae* family are assigned to one of the seven genera namely *Becurtovirus, Begomovirus, Curtoviurs, Eragrovirus, Mastrevirus, Topocuvirus* and *Turncurtovirus* (Varsani *et al*., 2009). On the other hand, depending on components of their genome, they can be sub-grouped into monopartite (one circular ssDNA genome) or bipartite (two circular ssDNA genomes of similar size) (King *et al*., 2012).

Virus species in the genus *Mastrevirus* have a monopartite genome approximately 2.7 kb encapsidated in geminate particles encoding four proteins separated by two intergenic regions (long intergenic region [LIR] and short intergenic region [SIR]). Both origin of replication and transcription start sites of the genome are located in the

LIR, while the SIR is the location of both the complementary-strand replication origin and transcription termination sites (Fig. 4.1).

The virion sense strand encodes two proteins; the movement protein (MP), functioning in cell to cell movement, and the coat protein (CP), which encapsidates the virion sense ssDNA and acting as the nuclear shuttle protein (NSP) for viral DNA. The complementary sense strand encodes the replication associated proteins Rep and Rep A (King *et al.*, 2012). Rep protein is expressed from spliced transcripts encompassing the two complementary sense strand ORFs C1 and C2 whereas Rep A is encoded from the C1 ORF of unspliced transcripts (Wright *et al.*, 1997).

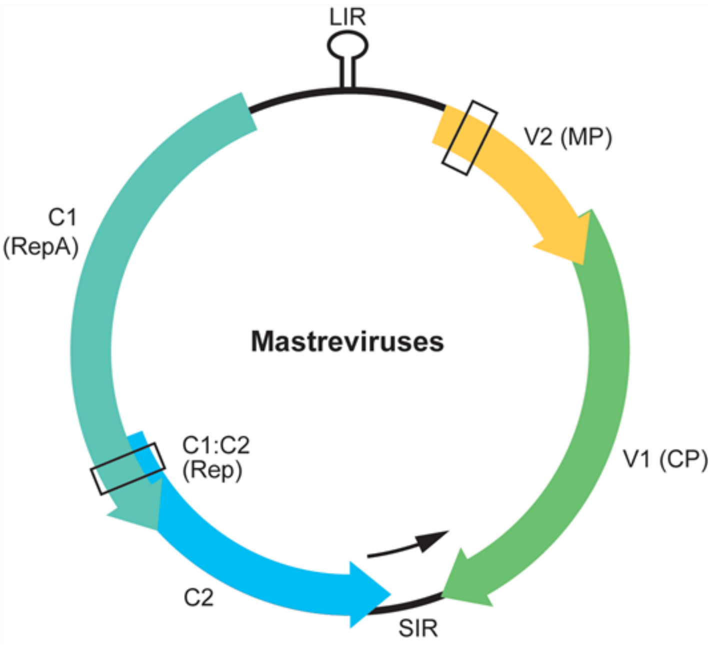


**Figure 4.1** Genomic organization of mastreviruses. ORFs are denoted as being encoded on the virion-sense (V) or complementary-sense (C) strand, and corresponding protein products are indicated. The positions of the stem-loop motif containing the conserved TAATATTAC sequence in the large intergenic region (LIR) and the encapsidated complementary-sense primer-like molecule (small arrow) in the small intergenic region (SIR) are shown. Introns (open boxes) occur in ORF V2 of some mastreviruses and at the overlap between ORFs C1 and C2 of all mastreviruses. CP, coat protein; MP, movement protein; Rep, replication- associated protein. Source: King *et al.*, 2012.

Virus species in the genus *Mastrevirus* are identified and discriminated by their genome sequences with a nucleotide threshold of < 78% identical sequences to other viruses indicating for distinct virus species and < 94% identical genome sequences considered as strain demarcation threshold (Zerbini *et al*., 2017). Three *Mastrevirus* species have been reported in maize. MSV and *Maize streak reunion virus* (MSRV) (Pande *et al*., 2012) are typical African viruses. Recently, *Maize striate mosaic virus* (MSMV) is described from Brazil (Fontenele *et al*., 2018).

MSV is the most prevalent maize virus in Africa and impacts maize production in Sub-Saharan Africa (Martin and Shepherd, 2009). Eleven strains of MSV, MSV-A to K in alphabetical order have been described (Muhire *et al*., 2013) with MSV-A strain being most widely distributed in maize production regions of Africa (Harkins *et al*., 2009). The second emerging and highly divergent *Mastrevirus* is MSRV. It has first been reported from maize in 2012 from La Réunion Island (Pande *et al*., 2012). Later it was reported from two uncultivated grass species, *Setaria barbata and Rottboellia* sp from Nigeria (Oluwafemi *et al*., 2014) and from maize in China (Chen *et al*., 2015) indicating its wider geographical distribution and host range.

In Ethiopia, a range of maize viruses have been described including MSV (Mesfin Tessera *et al*., 1991; Alemu Lencho *et al*., 1997), *Maize chlorotic mottle virus* [MCMV] and *Sugar cane mosaic virus* (SCMV) (Mahuku *et al*., 2015b; Menigstu Fantahun *et al*., 2017; Demsachew Guadie *et al*., 2019) and *Maize yellow mosaic virus* (Demsachew Guadie *et al*., 2018). Limited research has been done on characterizing maize mastreviruses in Ethiopia with only partial nucleotide sequence available at GenBank for an Ethiopian MSV isolate (X71956, (Briddon *et al*., 1994)).

This study was, therefore, designed to identify and analyze the genetic diversity of mastreviruses in major maize growing regions of the country. Moreover, lack of readly available antibodies, except for MSV, and to exploit the power of molecular methods to analyse our samples for other mastrevirus species including novel ones, motivated us to use RCA.

## 4.2 Material and methods

### Sample collection

The samples analyzed in this study were collected during the monotonous field survey of maize fields from five major maize growing regions of Ethiopia (Appendix III). Samples showing symptoms including broken or parallel streaks, fine streaks, and dwarfing were collected and dried over anhydrous calcium chloride. Eighty nine samples were selected from aggregates of samples collected in four rounds of survey from September 2015 (11 samples), in July 2016 (47), in November, 2016 (10) and in March 2017 (21) from major maize growing regions including Amhara (10), Benishangul-Gumuz (11), Oromia (45), South Nations, Nationalities and peoples (SNNP) (12) and Tigray (11). The collected samples were stored at room temperature before analysis.

### Detection and analysis of full-length maize mastrevirus sequences by RCA

Total DNA was extracted from ~20 mg dried maize leaf samples (each of the 89) using Qiagen DNeasy plant mini kit, Germany, following the manufacturer's instructions. For a sub-set of DNA extracts (28 samples) were individually multiplied by RCA to amplify circular viral DNA genomes using Phi29 DNA polymerase

(Illustra TempliPhi$^{TM}$, GE Healthcare, Germany) essentially as described before (Owor *et al*., 2007; Shepherd *et al*., 2008).

To proceed with the isothermal incubation, RCA, initially 6 µl of reaction mixture in each PCR tube containing 5 µl sample buffer and 1 µl of DNA sample was heated for 3 minutes at 95 °C for complete denaturation and immediately cooled on ice for 10 minutes. After cooling, a reaction mix containing 5 µl reaction buffer and 0.2 µl phi29 DNA polymerase was pipetted to each tube. The isothermal incubation was programed at 30 °C for 18 h followed by a final incubation at 65 °C for 10 min. Twenty amplified DNA preparations and pUC19 cloning vector were subjected to single site restriction digestion with *Bam*HI, *Sph*I or *Pst*I enzymes (New England Biolabs) to yield unit length genomes.

The restriction digestion reaction was made in a reaction volume of 20 µl containing 4 µl of the RCA products mixed with 8 µl of master mix containing 5.8 nuclease free water, 1.2 µl 10x NEB buffer 3.1 and 1 µl *Bam*HI, *Sph*I or *Pst*I. The reaction incubation lasts for 2 hours at 37 °C. To dephosphorylate the vector at the 5’-end and prevent it from self-ligation, 1 µl of Alkaline Phosphatase, Calf Intestinal (CIP) (New England Biolabs) was added after an hour of incubation to the reaction mix containing pUC19. Restriction digested dsDNA RCA products were electrophoresed and recovered from 1% agarose gel using Nucleospin Gel and PCR clean up mini kit (Macherey-Nagel GmbH, Germany) as recommended in the manufacturer’s protocol.

Fragment sizes of about 2.8 kb, indicating for mastrevirus genome DNA were excised from 1% agarose gels, ligated in a pUC19 vector. The ligation reaction was made in a total reaction volume of 10 µl containing 5 µl of the respective genomic DNA, 2.5 µl pUC19, 1 µl 10x T4 DNA ligase buffer (NEB), 0.5 µl ligase enzyme and 1 µl nuclease free water. Nuclease free water was used in place of the genomic DNA for the negative control. The ligation was performed at overnight incubation of the reaction mix at 4 °C.

The ligated products were subsequently transformed into DH5α electrocompetent host cells following the procedure described in chapter two. The multiplied plasmids were recovered from overnight culture using Nucleospin plasmid extraction kit (Macherey-Nagel). The correct inserts of the multiplied clones were confirmed by restriction digestion. The reaction was performed in a total reaction volume of 15 µl containing 5 µl of the recombinant clone, 1.5 µl cut-smart buffer, 7.5 µl nuclease free water, 0.5 µl *Aat*II restriction enzyme and additional 0.5 µl restriction enzymes of *Pst*I, *Sph*I or *Bam*HI in three separate reaction tubes. The restriction enzyme, *Aat*II was used to digest about 500 bp from the cloning vector pUC19 because the insert and the vector have almost equal size and it was very difficult to distinguish the one with the insert and those which do not contain the insert with out this restriction digestion.

One recombinant clone from each replicate was bi-directionally sequenced with the universal M13 primers. The full-genome of inserts in 10 plasmid DNA preparations were Sanger sequenced by primer walking using the sequencing service at Helmholtz Centre for Infection Research, Genomanalytik, GMAK, Germany. To generate the full-genome sequence of the respective plasmid DNA, sequenced DNA fragments were edited, aligned and assembled using Geneious 10.2.3 (Biomatters LTD, NZ).

For sequence confirmation, a pair of abutting primers (F- 5’-GAGGATCCCTAGTTGTAATAGGACG-3’ and R- 5’-AGGGATCCTCTGTTTTTTGACATGC-3’) was designed for end to end amplification of each novel genome the circular genome from DNA extracts. The PCR was made in a 20 µl of final volume containing 10 µl of Phusion Flash Mastermix (Thermo Fisher Scientific), 0.5 µl from each of the forward or reverse primers, 2 µl DNA ( approximately 100 ng/µl diluted) and 7 µl nuclease free water. Thermal cycling was programed for 35 cycles of 98 °C for 10 S, 56 °C for 20 S and 72 °C for 3 minutes preceded by 98 °C of complete denaturation for 30 S. The final extension was adjusted at 72 °C for 5 minutes.

**Phylogenetic relationship**

A phylogenetic analysis was conducted with the genome sequences generated and also including mastreviruses reference genomes. Additionally, sequences of *Geminivirus* type species of the genera *Curtovirus* (NC_001412), *Topocuvirus* (NC_003825) and *Begomovirus* (NC_001439) were chosen as out groups to generate a phylogenetic tree. Clustal alignment was done using the online Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) to further subject aligned sequences to MEGA 7.0.26 using the Maximum Likelihood method (Kumar *et al*., 2016). Bootstrap with 1000 resampling was applied to generate a phylogenetic tree. MUSCLE alignment of the sequence demarcation tool (SDTv1.2) (Muhire *et al*., 2014) was used to generate a color coded matrix from pairwise similarity calculations. The Clustal aligned sequences were scanned with recombination detection program (RDP) 4.39 (Martin *et al*., 2015) for possible recombination of novel genome sequences.

### Screening for presence of maize mastrevirus

To study the distribution of the three identified mastrevirus and any other possible maize-infecting mastrevirus in our samples, primers were designed for universal amplification or specific detection of mastreviruses. An initial universal confirmation for presence of mastreviruses was followed by specific amplification of virus sequences to identify and discriminate species. All the DNA extracts of each sample were diluted to 100 ng/µl prior to PCR amplifications.

A pair of designed general primer (Gen F- 5’-GGNGTNARRACBSAGTGGAAGAA-3’ and Gen R- 5’-GARGAYTGGHTGAAGGWNATGACTCC-3’) flanking 442bp spanning the CP coding region of the genome of mastreviruses was designed and used to screen 89 samples collected in different years of survey from major maize regions of Ethiopia (Appendex III). The PCR conditions were set for 35 cycles of 94 °C for 10 s, 52 °C for 40 s, and 72 °C for 30 s preceded by 94 °C pre-heating for 5 min and with a final extension at 72 °C for 5 min.

Then all the positive samples were separately screened with the three mastrevirus-specific primers (for MSV; F- 5’-AGCTGATATTTGGAGGACAAGCT-3’ and R- 5’-AGCTTGTCCGCCGGGAGT-3’; MSRV F- 5’-CCGGCTCCTGATGTACCGG-3’ and R- 5’-GAAACAAAAACGCAGGTAGGG-3’; MSDV F- 5’-GTGGACAGCAATTATTAAAGGCA-3’ and R- 5’-CCTTTTCGGACGCCTATCTCC-3’). For these primers, the annealing temperature was raised to 57 °C. The regions which were amplified by the primer pairs were 472 bp for MSV, 576 bp for MSRV and 623 bp for MSDV.

## 4.3 Result

### Sample collection

As clearly observed during sample collection from maize fields, maize seedlings showed various symptoms (Fig. 4.2). On one hand those seedlings infected with MSV showed clear broken or parallel white streaks throughout the leaf lamella (Fig. 4.2C) and those which are infected with MSRV showed yellowish strips (Fig. 4.2B). Those seedlings infected with the novel species however, showed dwarfing as well as very fine streaks and vein enation (Fig. 4.2A).

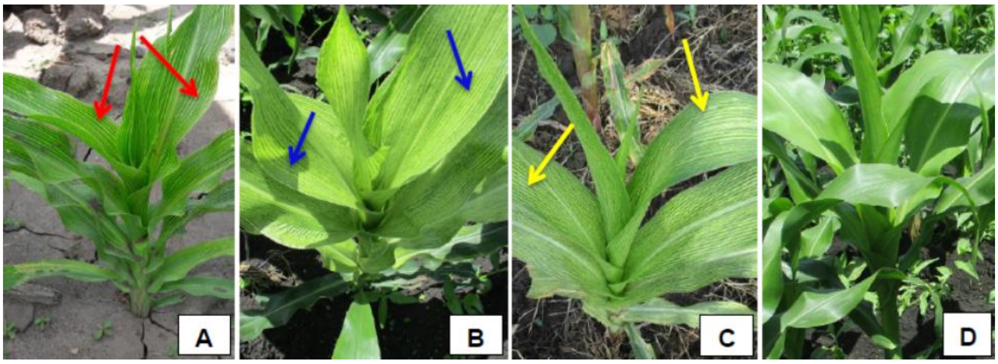


**Figure 4.2** Symptoms developed by maize seedlings as a result of single infection with MSDV (A), MSRV (B), MSV (C) and negative for the three viruses (D) as observed during field survey. Symptoms developed as a result of infections by each virus species are indicated by arrows.

### Detection of mastreviruses genomes using Rolling Circle Amplification

After confirmation of the recombinant clones with restriction digestion (Fig. 4.3), ten clones with DNA fragments of sizes similar to full-length genome sequences were sequenced. Annotation of the sequences revealed typical mastrevirus genes, LIR, SIR and the typical nonanucleotide sequence (Zerbini *et al*., 2017). BLASTN (Altschul *et al*., 1997) analysis of these sequences resulted in three distinct species of mastrevirus. In the first category, four MSV variants (Acc.Nos.MK329306 to MK329309) of the A-strain of MSV were identified. In the second group, two isolates (MK329304 and

MK329305) of MSRV were determined and in the last one four isolates (MK329300 to MK329303) of a novel mastrevirus species, with unusual nonanucleotide sequence has been described. The full-genome of MSV, MSRV and the novel species isolates determined in this study consist of 2687, 2879 and 2846 nucleotides, respectively.

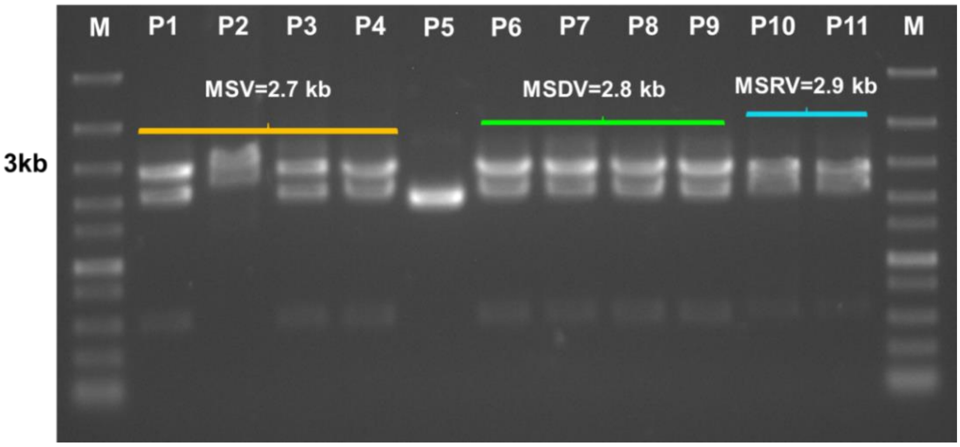


**Figure 4.3** Restricton digestion products of recombinant clones of the genomes of MSV, MSRV and MSDV. Lanes 1 to 3 were restriction digestion products of *Pst*I and *Aat*II, lanes 4 to 9 were digestion products of *Sph*I and *Aat*II and lanes 10 and 11 were digestion products of *Bam*HI and *Aat*II. The lower fent bads of about 500 bp are the restriction digestion products of *Aat*II from the pUC19 cloning vector. Fast DNA ladder was used as a size marker.

### Phylogenetic relationship

Phylogenetic analysis of the genome sequences and further 50 reference sequences retrieved from GenBank grouped the virus sequences of this study into three distinct clusters. The first cluster of four clones (MK329306 to MK329309) showed high nt identity with A-strain of MSV (NC_001346) (Fig. 4.4). These sequences shared a 98-100% identity and similarly were 97-98% identical to the reference sequence of MSV-A. Moreover, 80-90% identity with other strains of MSV (MSV-B to MSV-K) and a 56-70% identity with other species confirmed these sequences as MSV-A virus isolates (Fig. 4.5).

The second sequence cluster comprising two virus sequences (MK329304 and MK329305) was highly identical (96%) with a MSRV genome sequence (NC_017917) indicating that MSRV is present in Ethiopian maize.

Four sequences of virus DNA clones (MK329300 to MK329303) shared 99-100% sequence identity, however only distant sequence relationships were found with other mastreviruses. From BLASTN analysis, we observed that these isolates shared a maximum of 71% identity with Yunnan isolate (KT717933) of MSRV from China, and similarly, only a 70-71% identity with all the available sequences of MSRV. A pairwise comparison of CP and Rep amino acid sequences showed maximum identities of 63% and 68%, respectively, with isolates of MSRV (data not shown).

The limited (< 78% identical nt) genome identities recorded for these sequences forming a new clade well separated from MSRV (Fig. 4.4) fulfill the species demarcation criteria proposed by Muhire *et al*. (2013) and thus can be considered as distinct mastrevirus species. We also observed streak and dwarfing symptoms during the field survey (Fig. 4.2A), and this novel virus species is provisionally named as maize streak dwarfing virus (MSDV).

Three isolates of MSDV have a genome size of 2846 nt (the fourth one 2844 nt). These sequences are distantly related with MSV and MSRV genomes and have typical mastrevirus genome organization. Only the conserved nonanucleotide sequence TAATATT↓AC is diverse in MSDV with nucleotide mutations at the fourth and eighth positions (underlined, TAACATT↓GC) of this most conserved sequence in geminiviruses. Interestingly this unusual feature is also evident in *Sweet potato* *symptomless virus* 1 (SPSMV-1) with a nonanucleotide mutation at positions four and eight resulting in TAAGATT↓CC motif (Cao *et al*., 2017). Scanning of aligned sequences with recombination detection program (RDP) 4.39 (Martin *et al*., 2015) for any possible recombination failed to reveal any evidence that MSDV is recombinant. Sequences used in the comparison were diverse and most of them were distantly related (Fig. 4.6).

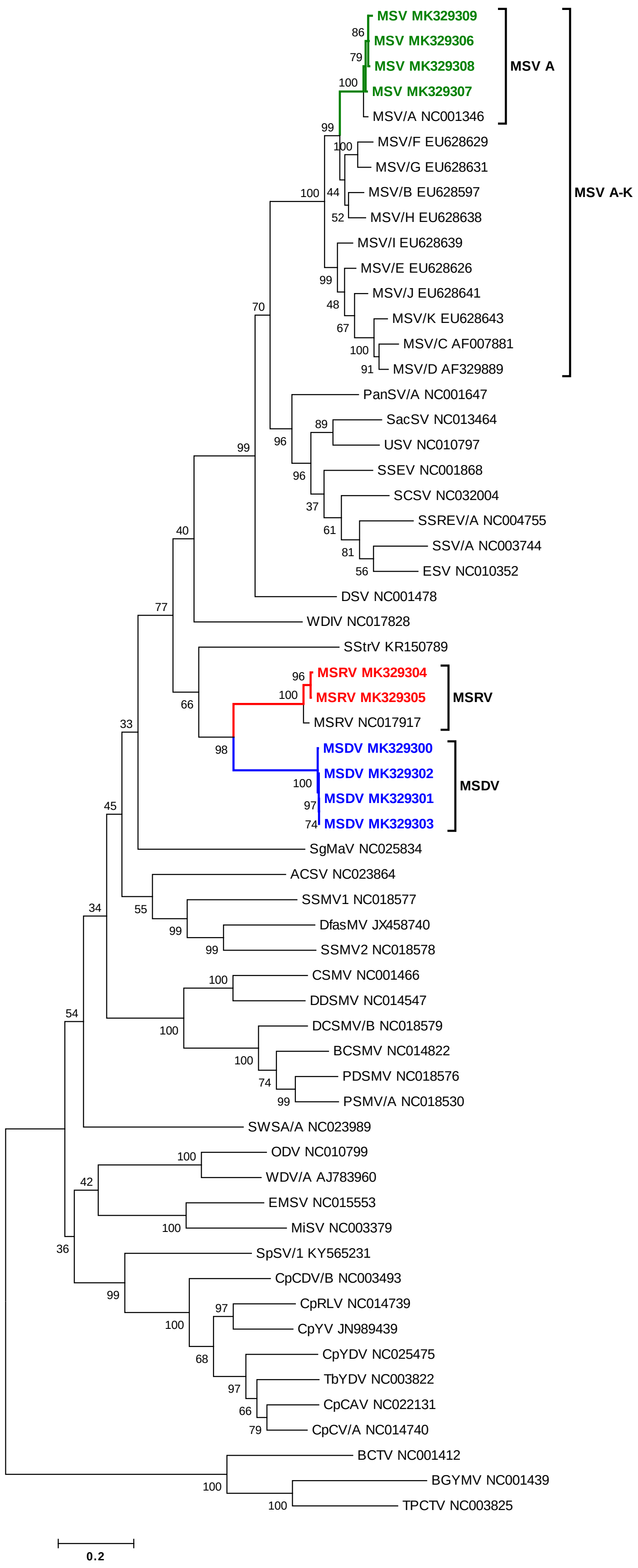


**Figure 4.4** Phylogenetic tree generated by the Maximum Likelihood method of MEGA7.0. based on full-genome sequence alignment of reference sequences in the genus *Mastrevirus*. Ten isolates determined in this study and 50 isolates (47 mastrevirus and three out groups representing type species from three genera of the *Geminiviridae* family) retrieved from the GenBank were used in the analysis. In those species with more than one strain, only the type species reference sequence was used except for MSV. Isolates determined in this study are indicated in colored and bold fonts. Species/Strain names and accession numbers are shown in the tree.

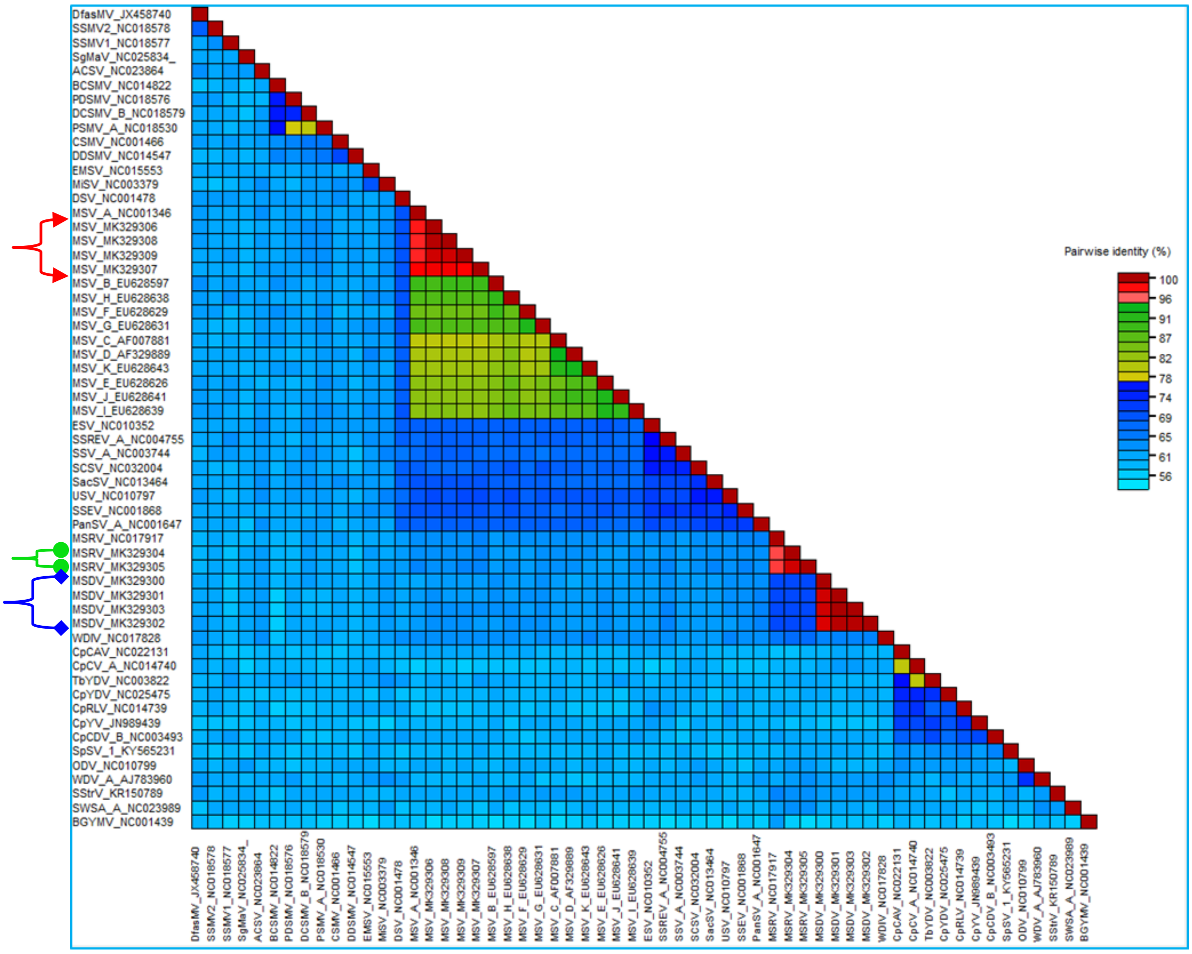


**Figure 4.5** Two dimensional pairwise alignment nucleotide identity plot of 60 full genome sequences of 57 mastrevirus species (10 determined in this study and 47 from GenBank) and three out group sequences from the three genera *Curtovirus*, *Topocuvirus*, *Begomovirus* in the *Geminiviridae* family (A); coat protein (CP) (B) and replication associated protein (Rep) (C) deduced using the MUSCLE alignment tool of the SDTv1.2 program. Each colored key represents a percentage to the identity score between two sequences.

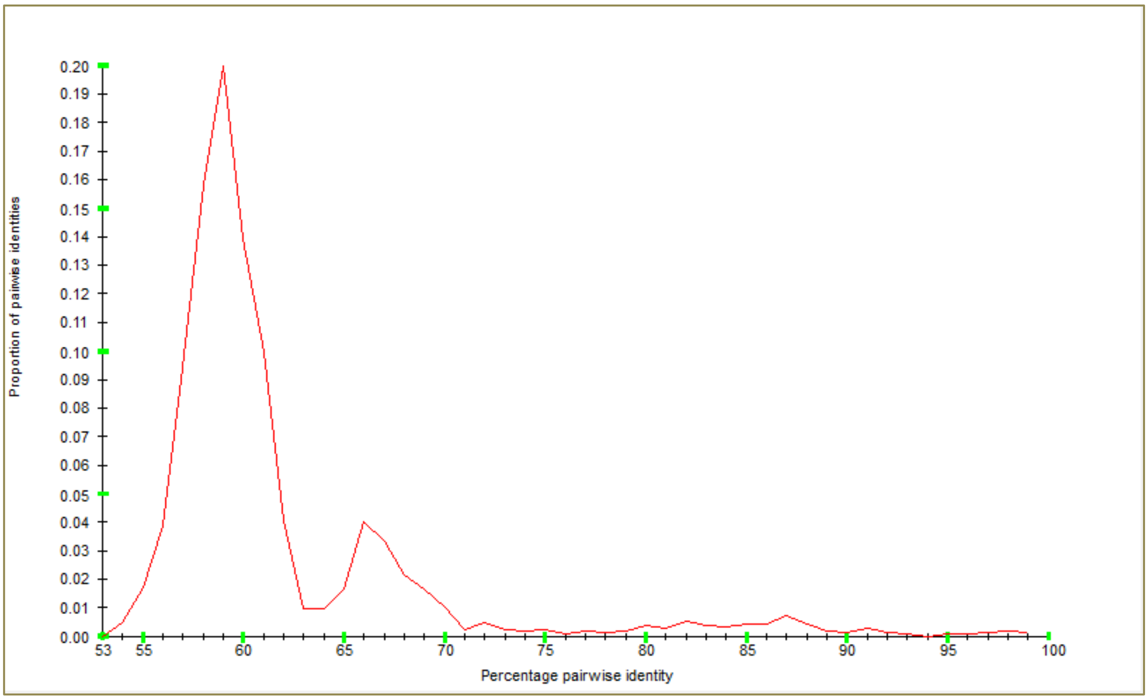


**Figure 4.6** A plot displaying the distribution of pairwise identity scores of 60 full genome sequences; of 57 mastrevirus species (10 determined in this study and 47 from GenBank) and three out group sequences from the three genera *Curtovirus*, *Topocuvirus*, *Begomovirus* in the fd*Geminiviridae* family.

## Distribution and incidence of mastreviruses infecting maize in Ethiopia

Eighty nine samples collected from August 2015 to March 2017 in major maize growing regions of Ethiopia, Amhara (10), Benishangul-Gumuz (11), Oromia (45), South Nations, Nationalities and peoples (SNNP) (12), and Tigray (11) were subjected to PCR screening. Of the 89 DNA aliquots screened with general mastrevirus primers, 50 of them were positive. A further screening of these DNA samples with specific primers for MSV, MSRV, and MSDV revealed 35 MSV, 13 MSRV and 11 MSDV sequences. Viruses were also found in mixed, double, as well as triple infections (Table 4.1). The most widely distributed virus, MSV was found in all surveyed regions. MSRV and MSDV were found in samples from Benishangul-Gumuze, Tigray, SNNP and Oromia regions. None of the samples from Amhara region were positive for MSRV and MSDV.

As revealed from PCR results, both MSV (100%) and MSRV (36%) have higher incidence in samples collected during August 2015 (late main rain feed cropping season) but MSDV (15%) was higher in samples collected during July 2016 (middle of main rain feed cropping season). On the other hand, the lower incidence was recorded for MSV (10%) during November 2016 (beginning of dry season, irrigation) and for MSRV and MSDV both 10% during March 2017 (middle of dry season).

When maize plants with clearly identified virus infections were examined for disease phenotypes, there were obvious differences in symptoms from MSV, MSRV and MSDV infections. Seedlings infected with MSV showed clear broken or parallel white streak symptoms (Fig. 4.2C). Infections with MSRV resulted in milder and less-pronounced yellowish strip symptoms (Fig. 4.2B) while seedlings infected with MSDV, the newly identified species were stunted with fine continuous or broken streaks across the leaf lamella and thickening of midveins (Fig. 4.2A).

**Table 4.1** Incidence and distribution of mastreviruses in PCR-screened samples from five major maize-growing regions of Ethiopia.

| No. | Region[a] | Year of collection | No. Samples | General Primer | MSV (+) | MSRV (+) | MSDV (+) | MSV + MSRV(+) | MSV + MSDV(+) | MSRV+ MSDV (+) | MSV + MSRV + MSDV (+) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Amhara | March 2017 | 10 | 1 | 1 | | | | | | |
| 2 | Benishangul-Gumuz | September 2015 | 7 | 7 | 7 | 3 | 1 | 3 | 1 | 1 | 1 |
| | | July 2016 | 4 | 4 | 2 | 2 | | | | | |
| 3 | Oromia | September 2015 | 4 | 4 | 4 | 1 | | 1 | | | |
| | | July 2016 | 31 | 12 | 6 | | 6 | | | | |
| | | November 2016 | 10 | 4 | 1 | 3 | 1 | 1 | | | |
| 4 | SNNP | July 2016 | 12 | 11 | 11 | 2 | 1 | 2 | 1 | | |
| 5 | Tigray | March 2017 | 11 | 7 | 3 | 2 | 2 | | | | |
| | | **Total** | **89** | **50(56%)** | **35(39%)** | **13(15%)** | **11(12%)** | **7(8%)** | **2(2%)** | **1(1%)** | **1(1%)** |

[a]SNNP = South Nations, Nationalities and People

## 4.4 Discussion

In this study, streak diseases of maize from Ethiopia were subjected to molecular analysis to identify viruses associated with the disease and characterize their genomes. The widely distributed MSV-A strain of MSV was also the most prevalent virus in the fields across all regions. As a new disease for Ethiopia, we present the occurrence of MSRV and, in addition, we provide evidence for the presence of a new, hitherto unknown mastrevirus species, tentatively named maize streak dwarfing virus (MSDV). The mastreviruses were found at least in some cases, in sympatry also causing mixed infections in the same host. As this paper is only the first comprehensive report, according to the best of our knowledge, describing different species of viruses causing maize streak disease, we present further investigation on the biology of the viruses to support the assessment on disease management options of a new disease situation.

The identification of MSV from maize in Ethiopia dates back to 1990's, and there is only limited genome sequence information available to date (Briddon *et al*., 1994) implies there is dearth of genomic sequence information of Ethiopian isolates. There is high degree of similarity between the MSV isolates determined in our study and that of other reference sequences, indicating highly conserved genomes of MSV-A isolates having a broad geographical distribution (Redinbaugh and Zambrano 2014; Yahaya *et al*., 2017a) and diverse hosts (Oluwafemi *et al*., 2014; Yahaya *et al*., 2017a and Zerbini *et al*., 2017) which may serve as an overwintering host throughout the year.

After its first report from maize in La Reunion Island (Pande *et al*., 2012), MSRV was also reported from two uncultivated grass species from Nigeria (Oluwafemi *et al*., 2014) and from maize in China (Chen *et al*., 2015). Here we report the occurrence of MSRV from Ethiopia for the first time. While this is a virus new to maize in Ethiopia, pending biological and ecological evidence, it is too early to speculate on impact of this virus to maize cultivation.

In addition, the new virus tentatively named MSDV is characterized by a divergent and unique genome and symptoms distinct from MSV or MSRV. While present in the region to almost similar extent like that of MSRV, there is no biological evidence to describe its impact on maize, consequences for epidemiology and even more, its origin and fate in the environment.

Interestingly, a divergent mastrevirus species, MSMV has been described from maize in Brazil (Fontenele *et al*., 2018). Pairwise comparison of full genome sequences of MSDV isolates described in our study with all the isolates of MSMV (MF167297-MF167307) showed < 61% identity, indicating that the two mastreviruses are only distantly related. This shows that new viruses appear to continuously emerge in Africa. Continuous monocultures and introduction of genotypes from abroad along with new encountered phenomenon may contribute to this situation. Thus virus survey and monitoring is required to keep abreast of new situations.

In general, this study shows that in Ethiopia three divergent species of genus *Mastrevirus* are identified from maize. This may attributed to the fact that Ethiopia is one of the Vavilov's countries for center of origin of cultivated plants (Vavilov, 1926) such as cereal crops including the so called 'orphan crops' which may host different viral pathogens for the possible evolution of these mastreviruses through recombination. Moreover, maize after being originated in Central America, Mexico, it was introduced to West Africa in the early 1500s by Portuguese trader (Dowswell *et al*., 1996) and then to Ethiopia during the 16th to 17th century (Haffangel, 1961).

Hence, cultivation of maize for more than 500 years in Ethiopia, a country with high cereal crop diversity and diverse agroecological zones, may also contribute for the evolution of three distinct mastrevirus species to infect the same host. On the other hand, these mastreviruses may earlier infect and co-evolved with indigenous African grass species causing latent symptoms and when maize was introduced to Africa, they might have migrated to it via existing leaf hopper vectors and adapted maize as a host causing infections and serious disease. Therefore, this report unequivocally ignites the initiative to assess mastrevirus and other virus species harbored by cereal crops as well as other cultivated and uncultivated grass species in Ethiopia.

Comparing the distribution of the three mastrevirus species in Ethiopia, MSV was found in lowland areas of Amhara, Benishangul-Gumuz, Oromia, SNNP and Tigray regions with higher disease incidence in lowland areas of SNNP and Benishangul-Gumuz. MSRV was found distributed in four regions except Amhara and the incidence rate has similar fashion as that of MSV. Even though, it has lower incidence than MSV and MSRV, MSDV was found in low and mid altitudes, in some cases in highland areas of the four regions except Amhara. The disease incidence also showed seasonal variation. This may attribute to the seasonal conditions favoring the growth and distribution efficiency of vectors or sampling sites favorable for the onset of the respective viruses.

In conclusion, here we report three distinct species of mastreviruses infecting maize in the same country which indicates higher diversity of maize infecting mastrevirus in Ethiopia. The existence of three distinct mastrevirus species infecting maize in a restricted geographical location will have direct implications in future breeding programs for resistance to maize viruses. Therefore, maize breeding programs for resistance of mastrevirus in Ethiopia should take at least these three species into consideration. More surveys should be made for MSDV in the Indian islands and other African countries which are hotspots for mastrevirus and where, in most cases, maize is cultivated as a major food crop.

# Chapter 5

# 5. Molecular and whole genome sequence analysis of Maize yellow mosaic virus in Ethiopia

## Abstract

*Maize yellow mosaic virus* (MaYMV) is a *Polerovirus* in the family *Luteoviridae*. It was first described from maize in China. Soon after, it has also been reported from different countries of Africa, Asia and South America as well as from different species of hosts harboring it, indicating its broader geographical distribution and host range. These recent reports of MaYMV from different parts of the world derive the motivation to analyze maize leaf samples collected from three major maize growing regions of Ethiopia for MaYMV and other possible poleroviruses infecting maize. For this study a total of 47 leaf samples were screened with RT-PCR using general polerovirus primers embracing 325 nucleotides of the coat protein gene. The RT-PCR, result indicated that 78% (in 2015) and 71% (in 2016) collected samples were positive for MaYMV. Direct sequencing of the RT-PCR products confirmed ≥ 99% nt identity of all the Ethiopian sequences and shared 98% to 99% identity to the reference sequence of MaYMV, KU248489. To study the genome variability of MaYMV from Ethiopia, one full-genome and two near full-genome sequences were generated using Illumina MiSeq next generation sequencing platform. All the three generated sequences of MaYMV shared 99.6% nt identity to each other. The complete MaYMV genome sequence (5642 nt) (MF684369) shared 96.8% nt identity with the Chinese isolate (KU248489), 98.2% with the Nigerian isolate (KY684356) and 95.4% with the isolate from Ecuador (KY052793). This first report of MaYMV in Ethiopia provides evidence for its nationwide distribution and the prevalence of a virus which may occur in Ethiopia for some time but was overlooked in targeted surveys focusing on MLND and other prominent maize diseases.

## 5.1 Introduction

The family *Luteoviridae* consists of three genra including genus *Luteovirus*, genus *Polerovirus* and genus *Enamovirus*. Viruses in this family contain a single molecule of linear, infectious, positive sense ssRNA genome of size ranging from 5.6 kb to 6.0 kb. The genome contains five or six open reading frames (ORFs) encoding proteins from 4 to 132 kDa. These RNA genomes lack a 3'-polyA tail and a small protein (VPg) is covalently linked to the 5' termini of *Poleroviruses* and *Enamovirus*. The 5' terminus of the genome of *Luteovirus* is experimentally proved to be phosphorylated (King *et al*., 2012).

The plant viruses belonging to the genus *Polerovirus* have six ORFs, ORF0 to ORF5 (Andrew *et al*., 2012). The genome contains three untraslated regions (UTRs) including an additional UTR between ORF2 and ORF3 besides the 5' UTR and 3' UTR (Mo *et al*., 2010; Krueger *et al*., 2013). The viral coat protein (CP) and movement protein (MP) are encoded by ORF3 and ORF4, respectively (Lee *et al*., 2002). A new ORF, termed ORF3a, was found located upstream of ORF3 and translated from a non-standard (not AUG) start codon, ACG. The protein encoded by ORF3a was reported to be involved for the long-distance movement of the virus in the host plant (Smirnova *et al*., 2015).

The first identified maize-infecting polerovirus was collected in Montana and identified as an isolate of barley yellow dwarf virus, BYDV-RMV, which was renamed maize yellow dwarf virus-RMV (MYDV-RMV) due to its host and vector specificity (Kruger *et al*., 2013). *Maize yellow mosaic virus* (MaYMV) is a recently described *Polerovirus* in the family *Luteoviridae*. It was first identified from maize (*Zea mays* L.) in China (Chen *et al*., 2016) displaying yellowing and mosaic symptoms. Later, it was reported from maize in Burkina Faso (Palanga *et al*., 2017), Brazil (Goncalves *et al*., 2017) and Ecuador (Bernreiter *et al*., 2017). It was also found infecting different hosts including sugarcane (*Saccharum* spp.) and itch grass (*Rottboellia cochinchinensis*) in Nigeria (Yahaya *et al*., 2017b) and *Panicum miliaceum* and *Sorghum bicolor* (Yoon *et al*., 2018) and maize (Lim *et al*., 2018) in South Korea; indicating its wider geographical distribution and versatility of the virus to infect a wide range of plant hosts. These recent reports of MaYMV from different host plant species in different countries provided the motivation to analyse our samples with general polerovirus primers and study the genome sequence variability of Ethiopian MaYMV isolates.

## 5.2 Materials and Methods

### Total RNA extraction and RT-PCR Screening of Maize leaf samples

To study the presence of MaYMV or other possible poleroviruses, we explored a collection of maize samples of local cultivars collected during virus surveys in August 2015 and July 2016 to assess the distribution of maize lethal necrosis disease (MLND) in three of the five major maize cultivation regions of Ethiopia including Benishangul-Gumuz, Oromia and South Nations, Nationalities and People (SNNP). The collection comprised a total of 47 leaf samples (Appendix IV) from symptomatic maize plants as dried tissues over anhydrous calcium chloride.

Total RNA was extracted from ~20 mg dried leaves using Epoch Life Science GenCatch Plant RNA purification Kit (Cat No.: 17-60250) following the manufacturer's instructions. Each RNA extract was screened by one step RT-PCR with the universal polerovirus primer pair Gen 1 (5'-CTCAARGCCTACCATGARTATAARATC-3') and Gen 2 (5'-CGTCTACCTATTTNGGRTTNTG-3'), comprising about 325 nucleotides of the coat protein gene (Afouda *et al*., 2017). Co-amplification of plant mRNA using the primer pair nad5-s (5'-GATGCTTCTTGGGGCTTCTTGTT-3') and nad5-as (5'-CTCCAGTCACCAACATTGGCATAA-3') for the nad5 gene (NADH dehydrogenase subunit 5) was included as an internal control for RNA degradation or RT-PCR inhibition for each reaction (Menzel *et al*., 2002).

The PCR reaction was performed in a one-step RT-PCR of total reaction volume 25 µl containing 2.5 µl 10x PCR buffer, 1.25 µl $MgCl_2$ (50 mM), 0.25 µl dNTP's (25 mM each), 1 µl total RNA extract, 1 µl from each of the forward and reverse polerovirus primers (10 µM), 0.2 µl *Moloney murine leukemia virus* (M-MLV) reverse transcriptase (200 U/µl), 0.1 µl Taq Polymerase (5 U/µl), 0.1 µl RiboLock RNase inhibitor (40 U/ µl) (Thermo Scientific), 0.25 µl from each of the forward and reverse *nad*5 primers (10 µM) and 17.1 µl nuclease free water.

Thermal cycling was programed as initial step of 42 °C for 60 minutes to synthesize cDNA and complete denaturation at 94 °C for 5 minutes followed by 35 cycles of 94 °C for 30 s, 54 °C for 40 S and 72 °C for 35 S. The final extension step was adjusted at 72 °C for 5 minutes. The PCR products were cleaned using Nucleospin Gel and PCR clean up mini kit (Macherey-Nagel GmbH, Germany) as recommended in the manufacturer's protocol and directly Sanger sequenced from both directions using the sequencing service at Helmholtz Center for Infection Research, Genomanalytik, GMAK, Germany for sequence confirmation.

### Illumina MiSeq Library Preparation and Full-genome Sequencing

To generate full-genome sequence and study genomic variability of MaYMV isolates from Ethiopia, total RNA extracts of three isolates collected from three provinces were selected to perform an RNA deep sequencing analysis. Three independent Illumina MiSeq libraries were prepared and were run on an Illumina MiSeq platform following the manufacturer's instructions as described by Afouda *et al*. (2017).

For transcriptome analysis, selective depletion of ribosomal RNA (rRNA) was done by treating 10 µl of each of the total RNA extract with RiboMinus Plant Kit (Life Technologies) following the manufacturer's instructions. This was followed by cDNA synthesis with a random octamer primer for library construction using the RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific). The reaction proceeded as follows: first, for complete denaturation, a total volume of 9.5 µl in each tube containing 1 µl random octamer primer, 0.5 µl RiboLock RNase inhibitor (20 U/µl) (Thermo Scientific), 2 µl total RNA extract, 1 µl dNTP mix (10 mM each) and 5 µl nuclease free water was incubated at 99 $^{\circ}$C for two minutes and immediately cooled on ice. Then a total volume of 10.5 µl containing 4 µl 5x reaction buffer, 1 µl RevertAid H Minus Reverse Transcriptase (200 U/µl), 0.5 µl RiboLock RNase inhibitor and 5 µl nuclease free water was pipetted in each tube containing the pre-denaturized RNA to generate a final volume of 20 µl.

For cDNA synthesis the thermal cycling program was adjusted at 25 $^{\circ}$C for 10 minutes, 42 $^{\circ}$C for 50 minutes and 85 $^{\circ}$C for 5 minutes. After purification the NEBNext mRNA Second Strand Synthesis Module (New England BioLabs Inc.) was applied according to manufacturer's instructions. Three independent paired-end Illumina libraries were prepared from the dscDNA with the Nextera XT Library Preparation Kit (Illumina, San Diego, CA, USA) for deep sequencing. Before sequencing, quality was checked by Qubit dsDNA High Sensitivity (HS) Assay Kit (Life Technologies) and Agilent HS DNA Kit (Agilent Technologies) and the liberaries were sequenced on an Illumina MiSeq platform with paired end reads (2x301) (DSMZ, Braunschweig, Germany).

### Sequence Analysis

The FASTQ files from Sanger sequencing output as well as the Illumina MiSeq next generation sequencing were analyzed. *De novo* and mapped based genome assembly was performed and contigs matching plant virus sequences were identified by BLASTN and TBLASTX against a custom plant virus sequence database based on NCBI sequences. Analysis was performed by Geneious v 10.2.3 by using the embedded Geneious assembler and mapper (Biomatters LTD, NZ).

Phylogenetic analysis was done with the genome sequences of MaYMV isolates determined in this study and those sequences retrieved from the GenBank. Sequence alignment was done using the online Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) and the aligned sequences were further subject to MEGA 7.0.26 using the Maximum Likelihood method (Kumar *et al*., 2016) and a bootstrap value of 1000 was applied to generate a phylogenetic tree.

## 5.3 Result

### Symptomatology and RT-PCR screening of samples

The collected leaf samples were showing yellowing, whitish to yellowish stripes and mosaic symptoms for MaYMV single infection for the tested viruses. More pronounced and sever disease symptoms were observed in samples infected with more than one virus in addition to MaYMV (Fig. 5.1). Co-amplification of plant *nad*5 gene mRNA with Maize yellow mosaic virus positive samples was observed in our samples (Fig. 5.2) confirming that the RNA extraction protocol was efficient and there was intact RNA available for RT-PCR. From the RT-PCR analysis of 47 samples, 72% (34 samples) were positive for the general polerovirus primers.

Regarding the disease distribution over year, 78% (seven from nine) and 71% (27 from 38) of the samples collected in 2015 and 2016, respectively were positive. Direct sequencing of the amplified DNA fragments confirmed ≥ 99% nt identity of all the Ethiopian sequences and shared 98% to 99% identity to the reference sequence of MaYMV (KU248489) from China (Chen *et al*., 2016). Whereas the shared identity to another maize infecting polerovirus species, Maize yellow dwarf virus RMV (MYDV-RMV, NC_021484) (Krueger *et al*., 2013), ranged from 80% to 81%.

Most of the samples used in the analysis (40 from 47) were positive at leats for one virus infection as revealed from ELISA test in chapter 3. From the remaining seven samples which were not positive for any of the viruses serologically tested in three of them were found infected with MaYMV single infection. The RT-PCR analysis result showed that 30% (14 from 47) were found in three viruses including MCMV, SCMV and MaYMV. There were also different combinations of double or triple infections (Table 5.1).

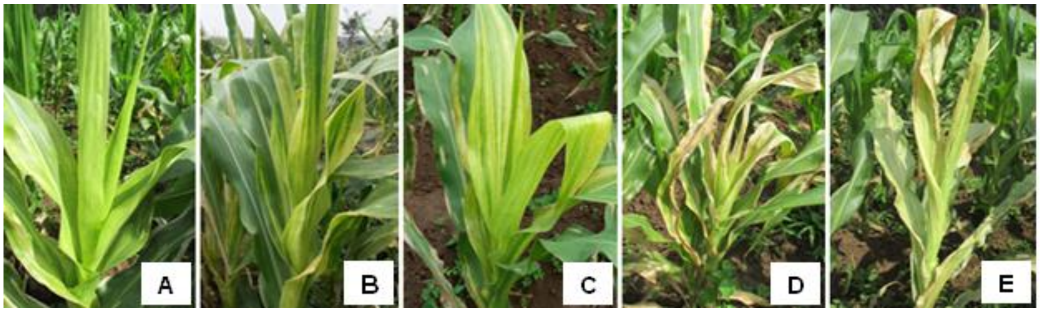


**Figure 5.1** Maize plants showing different symptomatology as a result of infections with MaYMV single infection (A); MaYMV and SCMV co-infection (B); MaYMV and MCMV co-infection (C); MCMV and SCMV co-infection (D) and MaYMV, MCMV and SCMV triple infection (E).

**Table 5.1** Distribution of Maize yellow mosaic virus in association with other viruses in ELISA and RT-PCR-screened samples from three maize-growing regions of Ethiopia.

| Region[a] | Year of collection | No. of samples | MaYMV | MaYMV + MCMV | MaYMV + SCMV | MaYMV + MSV | MaYMV + MCMV + MSV | MaYMV + MCMV + SCMV |
|---|---|---|---|---|---|---|---|---|
| Benishangul-Gumuz | August 2015 | 1 | | | | | 1 | |
| | July 2016 | 2 | 1 | | | | | |
| Oromia | August 2015 | 7 | | | | | | 5 |
| | July 2016 | 20 | 1 | 3 | 4 | | | 7 |
| SNNP | August 2015 | 1 | | 1 | | | | |
| | July 2016 | 16 | 1 | 3 | 2 | 1 | 2 | 2 |
| **Total** | | **47** | **3(6%)** | **7(15%)** | **6(13%)** | **1(2%)** | **3(6%)** | **14(30%)** |

[a]SNNP = South Nations, Nationalities and People

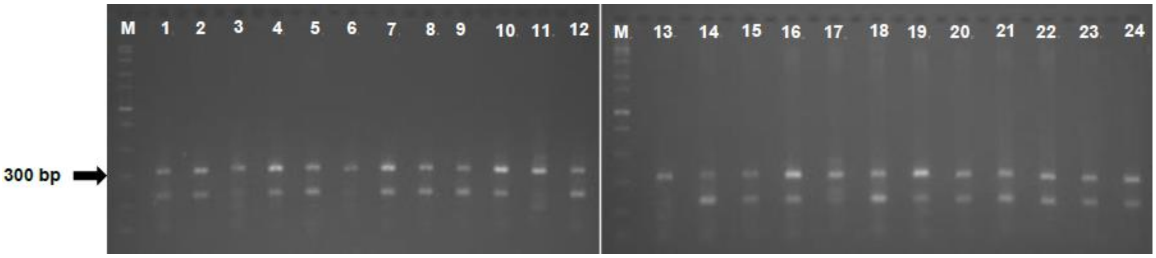


**Figure 5.2** Gel electrophoresis products of RT-PCR screening using general polerovirus primer (upper bands, 325 bp) and internal control part of *nad*5 gene primer pairs (lower bands, 181 bp). Fast DNA ladder was used as a size marker.

### Full-genome sequencing and analysis of the Ethiopian MaYMV isolates

The complete genome sequence of MaYMV isolate MV115 from Basha province, Oromia administrative region was determined to be 5642 nt (MF684369), which was assembled from 36,673 of 2,003,892 reads with an average coverage of 1141.7. Two near full-length genome sequences were determined, one for isolate MV32 from Welenchiti area (5498 nt, MF684367). This genome was obtained by assembling 1,820 reads from 2,517, 898 and mean coverage was 61.3). For isolate MV90 from Dugda Bora province (5504 nt, MF684368; 878 reads of 1,925,886 and 33.1 mean coverage).

The respective protein product of each open reading frame of our isolates follow similar fashion with other isolates of MaYMV described elsewhere (Fig. 5.3). The extreme 5’ and 3’ ends of the two near full-genome sequence isolates were not covered by the MiSeq Library. As a result isolate MV32 (MF684367) was missing 11 nucleotides from the 5’ end and MV90 (MF684368) missed 5 nucleotides from the same end. Both of the isolates were missing 134 nucleotides form the 3’ end.

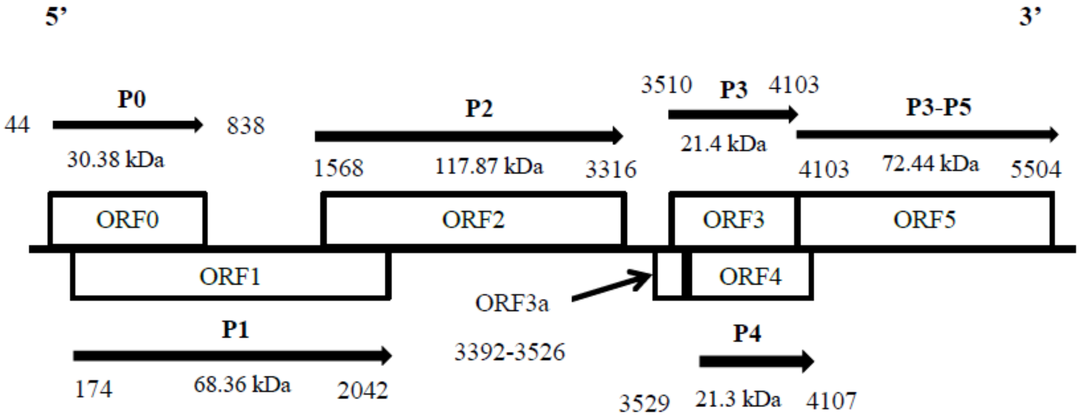


**Figure 5.3** Schematic representation of MaYMV, MV115 isolate (MF684369) genome organization. Boxes represent putative ORFs (ORF0 to ORF5). Protein products of each ORF (ORF0: putative viral suppressor of RNAi (VSR), ORF1: serine protease (Pro/VPg), ORF2: RNA-dependent RNA polymerase (RdRp), ORF3: coat protein (CP), ORF3a: putative long-distance movement factor, ORF4: movement protein (MP) and ORF5: coat protein read-through domain (RTD)) are indicated by lines with arrows (protein codes are above and calculated molecular masses are below the arrows).

### Sequence analysis and phylogeny

All the three full-genome and near full-genome sequences of MaYMV were registered to the GenBank (MF684367-MF684369) and shared > 99% identity score to each other and with those isolates from East Africa countries including Kenya and Tanzania and clustered in the same clade (Fig. 5.4A). These isolates showed slight variation when compared to the isolates from China (96 to 97%) and 95 to 96% identity with isolates from Equador and Brazil. Specifically the complete MaYMV genome sequence (5642 nt) of the Ethiopian isolate (MF684369) shared 96.8% nt identity with the Chinese isolate (KU248489), 98.2% with the Nigerian isolate (KY684356) and 95.4% with the isolate from Ecuador (KY052793) (Fig. 5.4B).

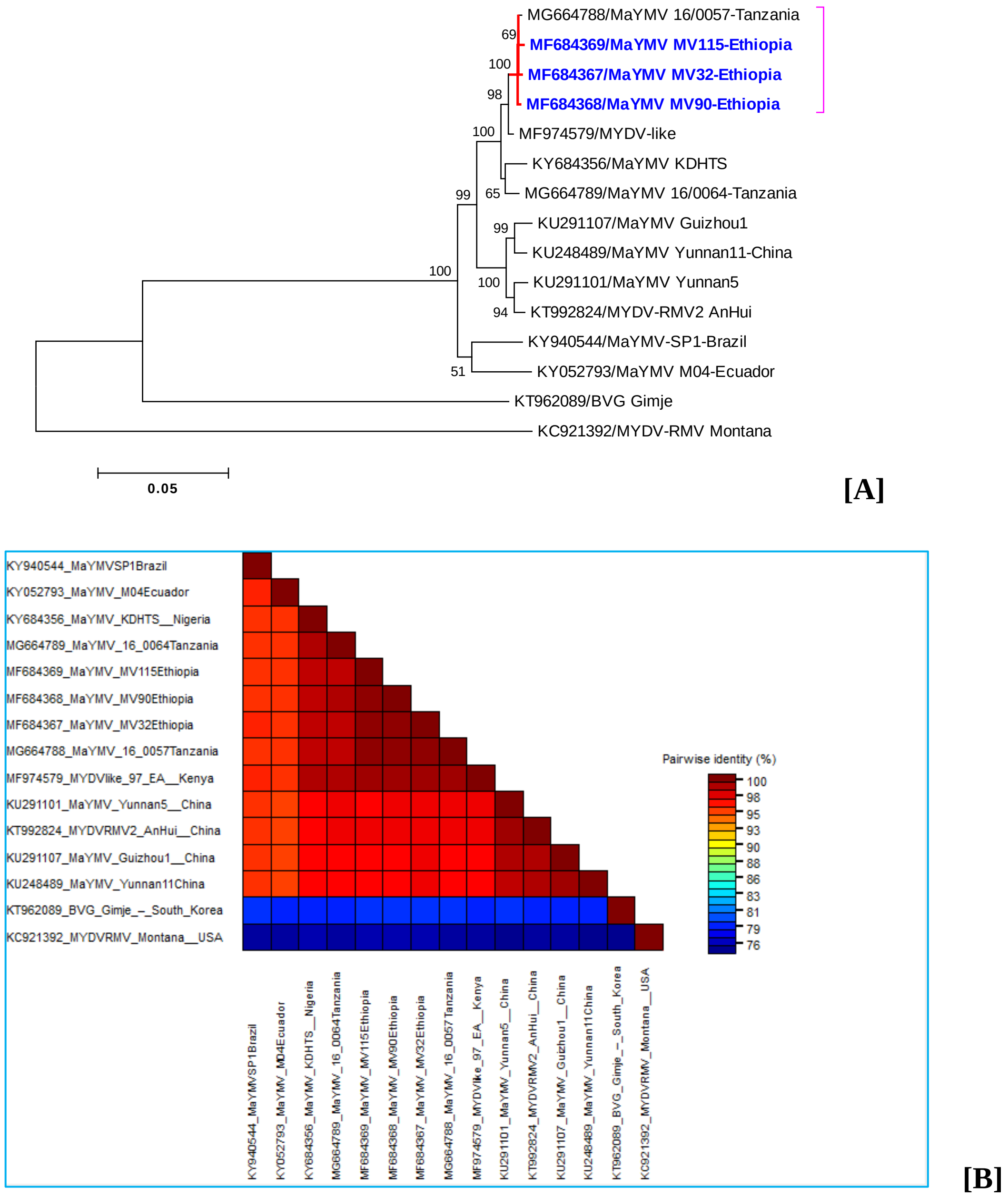


**Figure 5.4** Phylogenetic tree generated by the Maximum Likelihood method of MEGA 7.0. Fifteen full-genome sequences of MaYMV isolates (3 determined in this study and 12 retrived from GenBank) were used. Isolates determined in this study are indicated in blue bold fonts. GenBank accession number, isolate name and country of origin are shown in the tree for each isolate (A) and a color-coded sequence demarcation tool (SDT) matrix showing percent pairwise sequence identities (B).

## 5.4 Discussion

This first report of MaYMV in Ethiopia provides evidence for the distribution and prevalence of a virus which may occur in Ethiopia for some time but was overlooked in targeted surveys focusing on MLND and other prominent maize virus diseases. More than 70 % of the samples assayed in this experiment were positive for MaYMV which is an indication that more survey work is required to see its distribution at national level and develop integrated management options.

The complete genome sequence of the Ethiopian MaYMV isolate, having six ORFs is closely related to isolates from other countries which are associated with different plant species including maize, sugarcane, barley, itch grass (*Rottboellia cochinchinensis*), *Panicum miliaceum* and *Sorghum bicolor* (Chen *et al*., 2016; Goncalves *et al*., 2017; Palanga *et al*., 2017; Yahaya *et al*., 2017b; Lim *et al*., 2018; Yoon *et al*., 2018), indicating its wider geographical distribution and wide range of host species. This will make the management of this virus more difficult because it may use other species as an alternative or an overwintering host to escape difficult times.

Characteristic features of poleroviruses such as non-coding sequences between ORF2 and ORF3a (about 77 nucleotides) and the 'ACAAAA' consensus transcriptional start site in the 5′ end which is common in all poleroviruses were identified in our isolate, MV115. The frameshift from ORF1 into ORF2 occurs upstream of the termination of ORF1, and ORFs1 and 2 overlaping by 475 nucleotides. Presence of ORF4 (579 nucleotides) within

ORF3 and ORF5 which is the distinguishing feature of polerovirus genomes from those of the enamovirus is found in the genome of our isolates.

Adams *et al*. (2017) reported the co-occurrence of MaYMV with the commonly identified MLND disease complex viruses in maize leaf samples assayed from different East African countries. In line with this, analysis of our samples also indicated that more than 30% (14 from 47) are found in triple infection with MaYMV, MCMV and SCMV.

The clustering pattern of our isolates shows that they are closely related (> 98% identity) with the isolates from Tanzania, Kenya and Nigeria. This may indicate that our isolates have evolved from these related isolates and the virus has been circulating in Africa and lacks attention by focusing on surveys of other viruses.

Different combinations of double or triple infections were identified in the maize virus survey we conducted (Demsachew Guadie *et al*., 2019). More over, potential interactions of MaYMV with known MLN-causing viruses needs further study, including whether it is associated with or aggravate MLN by increasing the viral titer of MCMV or SCMV. Further research is required to: identify the possible vector (s) transmitting the virus; study yield loss caused by these viruses in single or different combinations of co-infections of maize with MaYM, and MCMV and/or potyviruses such as SCMV, MDMV, WSMV and JGMV as well as possible alternative hosts for MaYMV.

# Chapter 6

## 6. Conclusions and Recommendations

The rapid growth in population and urbanization will increase the demand for food as well as row materials for industries and other uses of maize in Ethiopia. Subsequently, maize will be one of the strategic crops to meet this demand. The emergence of new indigenous seed companies, importing seeds from abroad, coupled with new breeding strategies and the continued generation of productive hybrid varieties adapted to diverse production systems and socio-economic circumstances will enhance competitiveness of the seed system and contribute to sustained maize productivity in Ethiopia. In this regard, effective quarantine system and use of clean seed is crucial to manage circulation of disease causing agents across boarders. Moreover, factors limiting production and productivity of maize should be addressed and integrated management options should be developed. One of the limiting factors which render maize production is diseases caused by different virus species.

In this study attempts were made to survey maize fields from five major maize growing regions of Ethiopia and study the distribution, incidence and molecular diversity of maize associated RNA and DNA viruses. In the first section of the experiment, prior screening of our samples with DAS/TAS ELISA for eight common maize viruses revealed the presence of three species namely MCMV, SCMV and MSV. These viruses were found distributed almost in all of the surveyed regions in single or different combinations of co-infections. Maize lethal necrosis disease (MLND) caused by MCMV and SCMV co-infection was the most abundant disease in Oromia and SNNP regions whereas maize streak disease was the predominant virus infecting maize in the Benishangul-Gumuz region. Relatively low disease burden as a result of these viruses was observed from Amhara and Tigray regions.

Five of the viruses were not found in the tested samples. Therefore, these viruses may be absent or exist in trace incidence in maize fields of surveyed regions and escape sampling. Thus, they can be considered having less impact on maize production in Ethiopia.

As reported in previous works, there are different species of insect vectors transmitting MCMV from infected to healthy maize seedlings. There are also experimentaly confirmed alternative as well as overwintering hosts for this same species. Hence, inclusion and thorough assessment of these issues in future studies could have great role in breeding programs for virus resistance or integrated management system.

Molecular detection of maize leaf samples showing a variety of streak symptoms with RCA followed by cloning and sequencing revealed three distinct species of mastreviruses, MSV, MSRV and a novel species provisionally named maize streak dwarfing virus. PCR screening of about 89 smaples showing related symptoms with general and specific primers showed high distribution of these viruses in the maize regions of Ethiopia, MSV being incident followed by MSRV and the novel species in descending order. This indicates the potential of RCA technique to reveal different mastrevirus species including those undescribed before. Moreover, Ethiopia can also be considered as one of the potential area for diversity of maze-infecting mastreviruses. Potential vector species and alternative as well as overwintering hosts of these mastreviruses, especially MSRV and MSDV should be investigated for a better management of diseases caused by these viruses.

In future studies, it will be important to develop infectious clone for the mastrevirus species to study their biological properties, yield loss and other related concepts such as different combinations of double or tirple infections. The generic or species specific primers developed and used in this study can also be used in future ecological and/or epidimological studies as well as for screening seedlings during resitant line or infectious clone development.

The molecular analysis of MaYMV indicated high incidence and distribution of the virus in Ethiopia. Full-genome sequence analysis of this virus indicated little variability among the isolates and those described elsewhere in the world. MaYMV was also found in different combinations of MLND causative agents and other viruses such as MSV. In this regard, studies should be made if MaYMV has a role contributing for the disease complexity of MLND.

In general, use of different approaches of serological and/or molecular methods applied to samples collected in different growing seasons of the years from August 2015 to March 2017 from the maize growing regions of Ethiopia revealed a total of six virus species, three RNA and three DNA. Despite the obseravation of symptoms conspicuous of viral origin in almost all of our samples, no viruses were identified in 24% of 846 samples. This may be associated with the application of modern techniques only on some selected samples because the scope of this study didn’t allow us to screen all the samples with advanced techniques to reveal additional probable virus species. Though many virus species associated with maize were reported in previous studies, this work provides a comprehensive and up-to-date report about the species and distribution of maize viruses.

In conclusion, this piece of work reports maize associated viruses in Ethiopia, their incidence and distribution and calls for urgency for the integrated management of these viruses in Ethiopia to increase production and productivity of maize and there by contributing to food security in the country. Moreover, future maize breeding programs for resistance to viral diseases should consider at least these six species and others reported in previous studies.

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# Appendices

***Appendix* I.** List of Published articles from this thesis work in reputable journals.

1. Demsachew Guadie, Adane Abraham, Kassahun Tesfaye, Winter, S., Menzel, W. and Knierim, D. (2018). First report of maize yellow mosaic virus infecting maize (Zea mays L.) in Ethiopia. *Plant Dis*. **102**(5):1044.
2. Demsachew Guadie, Knierim, D., Winter, S., Kassahun Tesfaye, Adane Abraham (2019). Survey for the identification and geographical distribution of viruses and virus diseases of maize (*Zea mays* L.) in Ethiopia. *Eur. J. Plant Pathol*. **153**:271-281.
3. Demsachew Guadie, Knierim, D., Winter, S., Kassahun Tesfaye, Adane Abraham (2019). Molecular analysis of maize (Zea mays L.)-infecting mastreviruses in Ethiopia reveals marked diversity of virus genomes and a novel species. *Virus Genes* **55**(3):339-345.

***Appendix* II.** Buffer solutions used for ELISA test.

1. **Coating Buffer: per Litter (pH 9.6)**

   1.59 g sodium carbonate ($Na_2CO_3$)
   2.93 g sodium bicarbonate ($NaHCO_3$)
   0.20 g sodium azide ($NaN_3$)

Dissolve in 900 ml H2O, adjust pH to 9.6 with HCl and make up to 1 l.

2. **Phosphate Buffered Saline (PBS): per Litter (pH 7.4)**

   8.0 g sodium chloride (NaCl)
   0.2 g monobasic potassium phosphate ($KH_2PO_4$)
   1.15 g dibasic sodium phosphate ($Na_2HPO_4$)
   0.2 g potassium chloride (KCl)
   0.2 g sodium azide ($NaN_3$)

Dissolve in 900 ml H2O, adjust pH to 7.4 with NaOH or HCl and make up to 1 l.

**3. Washing Buffer: PBS-Tween (PBST): per Litter (pH 7.4)**

PBS + 500ul Tween 20

**4. Sample Extraction Buffer: per Litter (pH 7.4)**

PBST + 2% polyvinyl pyrrolidone (PVP)

**5. Conjugate Buffer: per Litter (pH 7.4)**

PBST + 2% PVP + 0.2% egg albumin

**6. Substrate Buffer: per Litter (pH 9.8)**

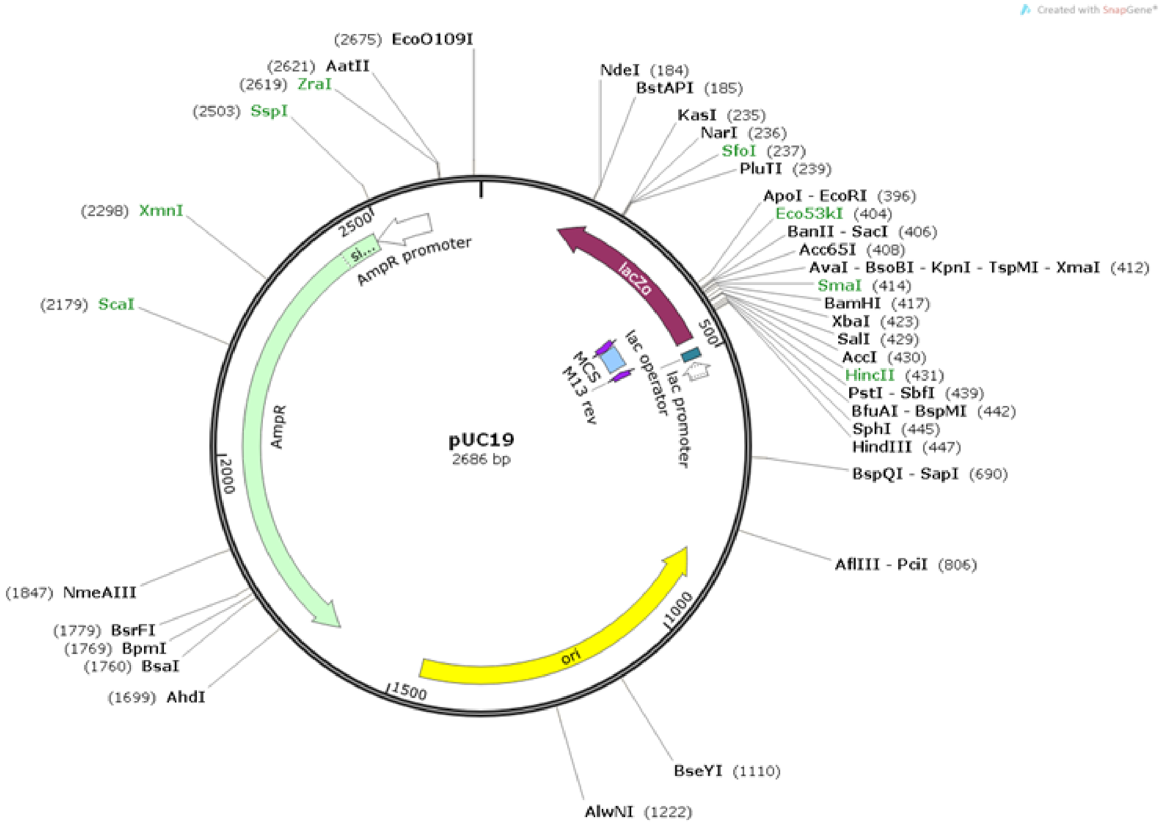
97 ml diethanolamine
800 ml distilled $H_2O$
0.2 g sodium azide ($NaN_3$)

Adjusted to pH 9.8 with HCl and make up to 1 liter with distilled $H_2O$

***Appendix* III.** Sequence registration information for partial or full-genomic sequences of viruses described in this study.

| **Serial No.** | **Sample Code** | **Site of collection** | **Date of collection** | **Accession Number** |
|---|---|---|---|---|
| ***Maize chlorotic mottle virus* (MCMV) Isolates coat protein gene sequences** | | | | |
| 1 | M 48 | Shire | March 2017 | MH286953 |
| 2 | MV 63 | Boset | July 2016 | MH286954 |
| 3 | MV 79 | Dugda Bora | July 2016 | MH286955 |
| 4 | MV 83 | Wondo | July 2016 | MH286956 |
| 5 | Et 111 | Pawe | August 2015 | MH286957 |
| 6 | Et 227 | Asosa | August 2015 | MH286958 |
| ***Maize streak virus* (MSV) Isolates coat protein gene sequences** | | | | |
| 7 | M 72 | Shire | March 2017 | MH286959 |
| 8 | M 93 | Fogera | March 2017 | MH286960 |
| 9 | Et 142 | Asosa | August 2015 | MH286961 |
| 10 | MV 166 | Gamo Gofa | July 2016 | MH286962 |
| 11 | MV 187 | South Omo | July 2016 | MH286963 |
| 12 | MV 260 | West Wollega | July 2016 | MH286964 |
| ***Sugar cane mosaic virus* (SCMV) Isolates coat protein gene sequences** | | | | |
| 13 | MV11 | Dera | July 2016 | MH286965 |
| 14 | MV 48 | Arsi | July 2016 | MH286966 |
| 15 | MV 63 | Boset | July 2016 | MH286967 |
| 16 | Et 68 | Wondo Genet | August 2015 | MH286968 |
| 17 | MV 79 | Dugda Bora | July 2016 | MH286969 |
| 18 | MV 83 | Wondo | July 2016 | MH286970 |
| ***Maize streak dwarfing virus* (MSDV) isolates full-genome sequences** | | | | |
| 19 | MV 1 | Adama Zuria | July 2016 | MK329300 |
| 20 | MV 56 | Shewaba | July 2016 | MK329301 |
| 21 | MV 66 | Boset | July 2016 | MK329302 |
| 22 | MV 74 | Analume | July 2016 | MK329303 |
| ***Maize streak Reunion virus* (MSRV) Isolates full-genome sequences** | | | | |
| 23 | MV 171 | Arbaminch Zuria | July 2016 | MK329304 |
| 24 | MV252 | Asosa | July 2016 | MK329305 |
| ***Maize streak virus* (MSV) Isolates full-genome sequences** | | | | |
| 25 | Et136 | Assossa | August 2015 | MK329306 |
| 26 | Et149 | Assossa | August 2015 | MK329307 |
| 27 | MV 171 | Arbaminch Zuria | July 2016 | MK329308 |
| 28 | MV242 | Berecha | July 2016 | MK329309 |
| ***Maize yellow mosaic virus* (MaYMV) Isolates full-genome sequences** | | | | |
| 29 | MV32 | Welenchiti | July 2016 | MF684367 |
| 30 | MV90 | Dugda Bora | July 2016 | MF684368 |
| 31 | MV115 | Basha | July 2016 | MF684369 |

***Appendix* IV.** The pUC19 cloning vector map and features.



***Appendix* V.** Pass-port data and results of 89 PCR screened samples with general and mastrevirus-specific primers.

| **No.** | **Code** | **Region**[a] | **Zone** | **Woreda** | **Collection Date** | **PCR Results**[b] | | | | **GPS Coordinates**[c] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | **General Primer** | **MSV Primer** | **MSRV Primer** | **MSDV Primer** | **Lat. (N)** | **Lon. (E)** | **Alt. (masl)** |
| 1 | M3 | Amhara | North Shewa | Shewa Robit | March 2017 | N | NT | NT | NT | 9.958 | 39.858 | 1375 |
| 2 | M10 | Amhara | North Shewa | Ataye | March 2017 | N | NT | NT | NT | 10.362 | 39.929 | 1501 |
| 3 | M25 | Amhara | North Wollo | Arbo | March 2017 | N | NT | NT | NT | 11.557 | 39.654 | 1640 |
| 4 | M33 | Amhara | North Wollo | Qobo | March 2017 | N | NT | NT | NT | 12.246 | 39.609 | 1454 |
| 5 | M35 | Amhara | North Wollo | Qobo | March 2017 | N | NT | NT | NT | 12.265 | 39.605 | 1460 |
| 6 | M84 | Amhara | South Gondar | Libokemkem | March 2017 | N | NT | NT | NT | 12.076 | 37.747 | 1835 |
| 7 | M85 | Amhara | South Gondar | Libokemkem | March 2017 | P | P | N | N | 12.076 | 37.747 | 1835 |
| 8 | M86 | Amhara | South Gondar | Libokemkem | March 2017 | N | NT | NT | NT | 12.076 | 37.747 | 1835 |
| 9 | M91 | Amhara | East Gojjam | Aneded | March 2017 | N | NT | NT | NT | 10.25 | 37.907 | 2352 |
| 10 | M93 | Amhara | East Gojjam | Aneded | March 2017 | N | NT | NT | NT | 10.252 | 37.909 | 2354 |
| 11 | Et136 | Benishabgul-Gumuz | Assossa | Assossa | September 2015 | P | P | N | N | 10.104 | 34.592 | 1597 |
| 12 | Et142 | Benishabgul-Gumuz | Assossa | Assossa | September 2015 | P | P | P | P | 10.212 | 34.479 | 1488 |
| 13 | Et149 | Benishabgul-Gumuz | Assossa | Assossa | September 2015 | P | P | N | N | 10.143 | 34.566 | 1542 |
| 14 | Et154 | Benishabgul-Gumuz | Assossa | Assossa | September 2015 | P | P | P | N | 10.192 | 34.562 | 1545 |
| 15 | Et166 | Benishabgul-Gumuz | Assossa | Assossa | September 2015 | P | P | P | N | 10.074 | 34.595 | 1541 |
| 16 | Et167 | Benishabgul-Gumuz | Assossa | Assossa | September 2015 | P | P | N | N | 10.074 | 34.595 | 1541 |
| 17 | Et170 | Benishabgul-Gumuz | Assossa | Assossa | September 2015 | P | P | N | N | 10.176 | 34.764 | 1480 |
| 18 | MV248 | Benishabgul-Gumuz | Assosa | Assosa | July 2016 | P | P | N | N | 10.043 | 34.568 | 1537 |
| 19 | MV251 | Benishabgul-Gumuz | Assosa | Assosa | July 2016 | P | N | P | N | 10.045 | 36.57 | 1539 |
| 20 | MV252 | Benishabgul-Gumuz | Assosa | Assosa | July 2016 | P | N | P | N | 10.045 | 36.57 | 1539 |
| 21 | MV254 | Benishabgul-Gumuz | Assosa | Assosa | July 2016 | P | P | N | N | 10.045 | 36.57 | 1539 |
| 22 | Et100 | Oromia | West Wollega | Gimbi | September 2015 | P | P | N | N | 9.191 | 35.804 | 1642 |
| 23 | Et121 | Oromia | West Wollega | Boji Dirmeji | September 2015 | P | P | N | N | 9.428 | 35.329 | 1908 |
| 24 | Et179 | Oromia | West Wollega | Mendi | September 2015 | P | P | P | N | 9.847 | 34.921 | 1433 |
| 25 | Et184 | Oromia | West Wollega | Mendi | September 2015 | P | P | N | N | 9.851 | 34.929 | 1612 |
| 26 | MV1 | Oromia | East Shewa | Adama Zuria | July 2016 | P | N | N | P | 8.467 | 39.304 | 1638 |
| 27 | MV3 | Oromia | East Shewa | Adama Zuria | July 2016 | N | NT | NT | NT | 8.467 | 39.304 | 1638 |
| 28 | MV6 | Oromia | East Shewa | Adama Zuria | July 2016 | N | NT | NT | NT | 8.467 | 39.304 | 1638 |
| 29 | MV14 | Oromia | Arsi | Dodota | July 2016 | N | NT | NT | NT | 8.302 | 39.323 | 1676 |
| 30 | MV17 | Oromia | Arsi | Dodota | July 2016 | N | NT | NT | NT | 8.301 | 39.323 | 1681 |
| 31 | MV19 | Oromia | Arsi | Dodota | July 2016 | N | NT | NT | NT | 8.268 | 39.293 | 1740 |
| 32 | MV21 | Oromia | East Shewa | Wolencheti | July 2016 | N | NT | NT | NT | 8.441 | 39.419 | 1340 |
| 33 | MV25 | Oromia | East Shewa | Wolencheti | July 2016 | N | NT | NT | NT | 8.441 | 39.419 | 1340 |
| 34 | MV27 | Oromia | East Shewa | Wolencheti | July 2016 | N | NT | NT | NT | 8.455 | 39.445 | 1327 |
| 35 | MV29 | Oromia | East Shewa | Wolencheti | July 2016 | N | NT | NT | NT | 8.455 | 39.445 | 1327 |
| 36 | MV30 | Oromia | East Shewa | Wolencheti | July 2016 | N | NT | NT | NT | 8.455 | 39.445 | 1327 |
| 37 | MV34 | Oromia | East Shewa | Wolencheti | July 2016 | N | NT | NT | NT | 8.469 | 39.461 | 1340 |
| 38 | MV37 | Oromia | East Shewa | Wolencheti | July 2016 | P | N | N | P | 8.486 | 39.523 | 1358 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 39 | MV40 | Oromia | East Shewa | Wolencheti | July 2016 | N | NT | NT | NT | 8.486 | 39.523 | 1358 |
| 40 | MV41 | Oromia | East Shewa | Wolencheti | July 2016 | N | NT | NT | NT | 8.486 | 39.523 | 1358 |
| 41 | MV56 | Oromia | Arsi | Shewaba | July 2016 | P | N | N | P | 8.662 | 39.751 | 1150 |
| 42 | MV64 | Oromia | East Shewa | Boset | July 2016 | P | N | N | P | 8.764 | 39.67 | 1243 |
| 43 | MV66 | Oromia | East Shewa | Boset | July 2016 | P | N | N | P | 8.764 | 39.67 | 1243 |
| 44 | MV67 | Oromia | East Shewa | Boset | July 2016 | N | NT | NT | NT | 8.764 | 39.67 | 1243 |
| 45 | MV71 | Oromia | East Shewa | Analume | July 2016 | N | NT | NT | NT | 8.624 | 39.391 | 1411 |
| 46 | MV72 | Oromia | East Shewa | Analume | July 2016 | N | NT | NT | NT | 8.624 | 39.391 | 1411 |
| 47 | MV73 | Oromia | East Shewa | Analume | July 2016 | N | NT | NT | NT | 8.431 | 39.029 | 1593 |
| 48 | MV74 | Oromia | East Shewa | Analume | July 2016 | P | N | N | P | 8.431 | 39.029 | 1593 |
| 49 | MV76 | Oromia | East Shewa | Analume | July 2016 | N | NT | NT | NT | 8.385 | 39.01 | 1596 |
| 50 | MV77 | Oromia | East Shewa | Analume | July 2016 | N | NT | NT | NT | 8.385 | 39.01 | 1596 |
| 51 | MV242 | Oromia | Illubabor | Berecha | July 2016 | P | P | N | N | 8.309 | 36.44 | 2139 |
| 52 | MV243 | Oromia | Illubabor | Berecha | July 2016 | P | P | N | N | 8.309 | 36.44 | 2139 |
| 53 | MV245 | Oromia | Illubabor | Jima Arjo | July 2016 | P | P | N | N | 8.708 | 36.464 | 1748 |
| 54 | MV258 | Oromia | West Wollega | Lalo Asabi | July 2016 | P | P | N | N | 9.209 | 35.723 | 1831 |
| 55 | MV259 | Oromia | West Wollega | Lalo Asabi | July 2016 | P | P | N | N | 9.209 | 35.723 | 1831 |
| 56 | MV260 | Oromia | West Wollega | Lalo Asabi | July 2016 | P | P | N | N | 9.209 | 35.723 | 1831 |
| 57 | V271 | Oromia | East Shewa | Adama | November 2016 | P | N | P | N | 8.416 | 39.325 | 1548 |
| 58 | V293 | Oromia | East Shewa | Adama | November 2016 | N | NT | NT | NT | 8.403 | 39.323 | 1541 |
| 59 | V325 | Oromia | East Shewa | Jeju | November 2016 | N | NT | NT | NT | 8.591 | 39.695 | 1185 |
| 60 | V326 | Oromia | East Shewa | Jeju | November 2016 | N | NT | NT | NT | 8.591 | 39.695 | 1185 |
| 61 | V331 | Oromia | East Shewa | Jeju | November 2016 | N | NT | NT | NT | 8.689 | 39.614 | 1133 |
| 62 | V334 | Oromia | East Shewa | Jeju | November 2016 | P | P | P | N | 8.689 | 39.614 | 1133 |
| 63 | V357 | Oromia | Arsi | Bole | November 2016 | P | N | N | P | 8.662 | 39.752 | 1147 |
| 64 | V358 | Oromia | Arsi | Bole | November 2016 | P | N | P | N | 8.662 | 39.752 | 1147 |
| 65 | V370 | Oromia | Arsi | Bole | November 2016 | N | NT | NT | NT | 8.706 | 39.744 | 1152 |
| 66 | V372 | Oromia | East Shewa | Zeway | November 2016 | N | NT | NT | NT | 7.948 | 38.717 | 1481 |
| 67 | MV149 | SNNP | Wolaita | Humbo | July 2016 | P | P | N | N | 6.652 | 37.816 | 1346 |
| 68 | MV166 | SNNP | Gamo Gofa | West Abaya | July 2016 | P | P | P | N | 6.026 | 37.669 | 1181 |
| 69 | MV168 | SNNP | Gamo Gofa | West Abaya | July 2016 | P | P | N | N | 6.026 | 37.669 | 1181 |
| 70 | MV169 | SNNP | Gamo Gofa | West Abaya | July 2016 | N | NT | NT | NT | 6.026 | 37.669 | 1181 |
| 71 | MV171 | SNNP | Gamo Gofa | Arbaminch Zuria | July 2016 | P | P | P | N | 6.061 | 37.563 | 1139 |
| 72 | MV172 | SNNP | Gamo Gofa | Arbaminch Zuria | July 2016 | P | P | N | N | 6.061 | 37.563 | 1139 |
| 73 | MV177 | SNNP | Gamo Gofa | Arbaminch Zuria | July 2016 | P | P | N | N | 6.061 | 37.563 | 1139 |
| 74 | MV182 | SNNP | Gamo Gofa | Arbaminch Zuria | July 2016 | P | P | N | N | 5.71 | 37.433 | 1182 |
| 75 | MV186 | SNNP | South Omo | South Ari | July 2016 | P | P | N | N | 5.738 | 36.611 | 1350 |
| 76 | MV187 | SNNP | South Omo | South Ari | July 2016 | P | P | N | P | 5.738 | 36.611 | 1350 |
| 77 | MV198 | SNNP | South Omo | Qey Afer | July 2016 | P | P | N | N | 5.634 | 36.674 | 1237 |
| 78 | MV232 | SSNP | Gurage | Abeshki | July 2016 | P | P | N | N | 8.255 | 37.625 | 1613 |
| 79 | M47 | Tigray | Mychew | Alamata | March 2017 | P | N | P | N | 12.48 | 39.605 | 1470 |
| 80 | M50 | Tigray | Mychew | Alamata | March 2017 | P | N | N | P | 12.48 | 39.605 | 1470 |
| 81 | M51 | Tigray | Mychew | Alamata | March 2017 | N | NT | NT | NT | 12.48 | 39.605 | 1470 |
| 82 | M52 | Tigray | Mychew | Alamata | March 2017 | P | N | P | N | 12.48 | 39.605 | 1470 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 83 | M65 | Tigray | Adigrat | Gantafash | March 2017 | N | NT | NT | NT | 14.27 | 39.305 | 2069 |
| 84 | M66 | Tigray | Adigrat | Gantafash | March 2017 | P | N | N | P | 14.27 | 39.305 | 2069 |
| 85 | M76 | Tigray | Shire | Medewayzana | March 2017 | P | P | N | N | 14.242 | 38.393 | 1785 |
| 86 | M77 | Tigray | Shire | Medewayzana | March 2017 | P | P | N | N | 14.242 | 38.393 | 1785 |
| 87 | M78 | Tigray | Shula | Tselemt | March 2017 | P | P | N | N | 13.594 | 38.15 | 1313 |
| 88 | M79 | Tigray | Shula | Tselemt | March 2017 | N | NT | NT | NT | 13.594 | 38.15 | 1313 |
| 89 | M81 | Tigray | Shula | Tselemt | March 2017 | N | NT | NT | NT | 13.546 | 38.144 | 1296 |

[a]Region SNNP = South Nations, Nationalities and People; [b]PCR Results P = Positive, N = Negative and NT = Not tested; [c]GPS Coordinates Lat. = Latitude, Lon. = Longitude, Alt. = Altitude

***Appendix* VI.** Pass-port data and PCR results of 47 samples screened with general polerovirus primer pairs.

| No. | Sample code[a] | Region[b] | Zone | Woreda (Province)[c] | Collection Date | Universal Polerovirus Primer PCR Results[d] | GPS Co-ordinates[e] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lat. (N) | Lon. (E) | Alt. (masl) |
| 1 | Et135 | Benishangul-Gumuz | Assossa | Amba 5 | August 2015 | P | 10.104 | 34.592 | 1597 |
| 2 | MV253 | Benishangul-Gumuz | Assossa | AARC on station | July 2016 | N | 10.045 | 36.57 | 1539 |
| 3 | MV249 | Benishangul-Gumuz | Assossa | AARC on station | July 2016 | P | 10.043 | 34.568 | 1537 |
| 4 | Et227 | Oromia | Arsi | Awash Bishola | August 2015 | P | 8.388 | 39.331 | 1541 |
| 5 | MV53 | Oromia | Arsi | Bole | July 2016 | P | 8.662 | 39.751 | 1150 |
| 6 | MV8 | Oromia | Arsi | Dera | July 2016 | N | 8.388 | 39.33 | 1534 |
| 7 | MV9 | Oromia | Arsi | Dera | July 2016 | P | 8.388 | 39.33 | 1534 |
| 8 | MV46 | Oromia | Arsi | Dera | July 2016 | N | 8.388 | 39.33 | 1534 |
| 9 | Et249 | Oromia | Arsi | Dodota | August 2015 | P | 8.374 | 39.329 | 1534 |
| 10 | Et198 | Oromia | Arsi | Dodota | August 2015 | P | 8.426 | 39.402 | 1345 |
| 11 | MV48 | Oromia | Arsi | Jeju | July 2016 | P | 8.519 | 39.461 | 1178 |
| 12 | MV44 | Oromia | Arsi | Jeju | July 2016 | P | 8.519 | 39.461 | 1178 |
| 13 | Et49 | Oromia | East Shewa | Adami Tullu | August 2015 | N | 7.951 | 38.699 | 1643 |
| 14 | MV264 | Oromia | East Shewa | Adama Zuria | July 2016 | P | 8.424 | 39.332 | 1552 |
| 15 | Et14 | Oromia | East Shewa | Adama Zuria | August 2015 | P | 8.425 | 39.318 | 1549 |
| 16 | MV69 | Oromia | East Shewa | Boset | July 2016 | N | 8.683 | 39.501 | 1307 |
| 17 | MV63 | Oromia | East Shewa | Boset | July 2016 | P | 8.764 | 39.67 | 1243 |
| 18 | MV58 | Oromia | East Shewa | Boset | July 2016 | P | 8.689 | 39.728 | 1186 |
| 19 | MV65 | Oromia | East Shewa | Boset | July 2016 | P | 8.764 | 39.67 | 1243 |
| 20 | MV90 | Oromia | East Shewa | Dugda Bora | July 2016 | P | 8.197 | 38.395 | 1638 |
| 21 | MV82 | Oromia | East Shewa | Dugda Bora | July 2016 | P | 8.367 | 39.005 | 1590 |
| 22 | MV96 | Oromia | East Shewa | Dugda Bora | July 2016 | P | 8.097 | 38.772 | 1652 |
| 23 | MV79 | Oromia | East Shewa | Malima Beri | July 2016 | P | 8.367 | 39.005 | 1590 |
| 24 | MV83 | Oromia | East Shewa | Malima Beri | July 2016 | P | 8.367 | 39.005 | 1590 |
| 25 | Et291 | Oromia | East Shewa | MARC on station | August 2015 | N | 8.436 | 39.316 | 1483 |
| 26 | Et305 | Oromia | East Shewa | MARC on station | August 2015 | P | 8.442 | 39.309 | 1565 |
| 27 | MV32 | Oromia | East Shewa | Welenchiti | July 2016 | P | 8.469 | 39.461 | 1340 |
| 28 | MV22 | Oromia | East Shewa | Welenchiti | July 2016 | P | 8.441 | 39.419 | 1340 |
| 29 | MV36 | Oromia | East Shewa | Welenchiti | July 2016 | P | 8.486 | 39.523 | 1358 |
| 30 | MV23 | Oromia | East Shewa | Welenchiti | July 2016 | N | 8.441 | 39.419 | 1340 |
| 31 | MV171 | SNNP | Gamo-Gofa | Arbaminch-Zuria | July 2016 | P | 6.061 | 37.563 | 1139 |
| 32 | MV178 | SNNP | Gamo-Gofa | Arbaminch-Zuria | July 2016 | P | 5.736 | 37.434 | 1156 |
| 33 | MV170 | SNNP | Gamo-Gofa | Arbaminch-Zuria | July 2016 | N | 6.061 | 37.563 | 1139 |
| 34 | MV175 | SNNP | Gamo-Gofa | Arbaminch-Zuria | July 2016 | N | 6.061 | 37.563 | 1139 |
| 35 | MV173 | SNNP | Gamo-Gofa | Arbaminch-Zuria | July 2016 | N | 6.061 | 37.563 | 1139 |
| 36 | MV176 | SNNP | Gamo-Gofa | West Abaya | July 2016 | N | 6.026 | 37.669 | 1181 |
| 37 | MV227 | SNNP | Gurage | Abeshge | July 2016 | P | 8.264 | 38.732 | 1816 |
| 38 | MV115 | SNNP | Hawassa | Basha | July 2016 | P | 7.093 | 38.623 | 1773 |

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|---|---|---|---|---|---|---|---|---|---|
| 39 | MV121 | SNNP | Hawassa | Basha | July 2016 | N | 7.094 | 38.626 | 1788 |
| 40 | Et68 | SNNP | Hawassa | Wondo Genet | August 2015 | P | 7.095 | 38.621 | 1789 |
| 41 | MV111 | SNNP | Hawassa | Wondo Genet | July 2016 | P | 7.014 | 38.59 | 1713 |
| 42 | MV141 | SNNP | Sidama | Sula | July 2016 | P | 7.057 | 38.517 | 1681 |
| 43 | MV142 | SNNP | Sidama | Sula | July 2016 | P | 7.057 | 38.517 | 1681 |
| 44 | MV204 | SNNP | South Omo | Ayke Dego | July 2016 | N | 5.477 | 36.958 | 547 |
| 45 | MV196 | SNNP | South Omo | Tsemey | July 2016 | P | 5.662 | 36.661 | 1267 |
| 46 | MV193 | SNNP | South Omo | Tsemey | July 2016 | P | 5.662 | 36.661 | 1267 |
| 47 | MV197 | SNNP | South Omo | Tsemey | July 2016 | P | 5.662 | 36.661 | 1267 |

[a]Sample code: MV= maize virus, Et = Ethiopia; [b]Region: SNNP= South Nations and Nationalities People; [c]province: AARC = Assossa Agricultural Research Center, MARK = Melkasa Agricultural Research Center; [d]PCR results: P = positive, N = negative; [e]GPS Co-ordinates: Lat. = latitude, Lon. = longitude, Alt. = altitude.