ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Determination of the Putative Insect Vector of Napier Grass Stunt Phytoplasma using Molecular and Bioassay techniques in Western Kenya

By

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A Thesis Submitted to the School of graduate studies of Addis Ababa University In partial fulfillment of the requirements for the Degree of Master of Science in Biology

July, 2008

Declaration of Originality

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

Evans Odhiambo Obura

"Could Hamlet have been written by a committee?
Or Mona Lisa painted by a club? Could the
New Testament have been composed as a
conference report? Creative ideas do not spring
from groups. They spring from individuals"

- A. Whitney Griswold

Acknowledgements

Overwhelming appreciation goes to my supervisors; Dr. Belay Gurja, Dr. Daniel Masiga, and Dr. ZR Khan for the patience, encouragement, interest and critical discussion they provided throughout this study. Donor funding was critical for this work. I therefore thank Dr. Ochieng Odero; the ARPPIS Network coordinator and the entire ARPPIS secretariat for providing funds which made it possible to pursue my studies through a scholarship award. A big salute goes to *Kilimo Trust foundation* for providing funds to support Ngs-phytoplasma vector research.

I deeply salute the work done by Lilian Auma and Ltilin Bargul, who optimized the working conditions of PCR primers for the detection of Napier Grass Stunt Phytoplasma (Ngs-phytoplasma). Their work provided insights into PCR phytoplasma. Field experiments were only possible through the efforts of Ishmael Kidiavai, Silas Ouko, Janet Odongo and the entire ICIPE community at Mbita point field station. My gratitude extends to all members of Molecular Biology and Biotechnology Department (MBBD) and the entire ICIPE community at Duduville in Nairobi, for providing a good environment conducive for research. Laboratory supplies were always available through James Kabii.

This work was only possible because of the role played by my Dear wife; Maureen Achieng who managed to run the home single-handedly while I was away for studies in Ethiopia. I cannot forget the part played by my children, brothers, sisters and the entire family, for the everlasting encouragement to soldier on. It was shoff here and a shove there, but, the great hands of Jehovah, the Lord of all hosts, took me through this endeavor successfully.

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List of abbreviations

µl	Microlitres
AAP	Acquisition Access period
ARPPIS	African Regional Post-graduate Programme in Insect Sciences
AFMTA	Artificial Feeding Medium Transmission Assay
BIP	Backward Inner Primer
bp	Base pairs
CTAB	Cetyltrimethylammonium Bromide
cv	Cultivar
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediamine tetracetic acid
ELISA	Enzyme linked immunosorbent assay
EM	Electron Microscopy
ESRI	Environmental Systems Research Institute
EtBr	Ethidium Bromide
FIP	Forward Inner Primer
GC	Guanine, Cytocine (nucleotide bases)
HCL	Hydrochloric acid
IAP	Inoculation Access Period
ICIPE	International Centre for Insect Physiology and Ecology
ICSB	International Committee for Systematic Bacteriology
ILRI	International Livestock Research Institute
in vitro	In an artificial environment, such as a test tube
LAMP	Loop mediated amplification
Mins	Minutes
ml	Milliliters
MLOs	Mycoplasma-like organisms
mM	Millimolar
NaCl	Sodium Chloride
HNC	Homoptera of Napier canopy

Ngs- phytoplasma	Napier grass stunt phytoplasma
Ngs-disease	Napier Grass Stunt disease
Ngs-phytoplasma-Ethiopia	Napier grass stunt Ethiopia
Ngs-phytoplasma-Kenya	Napier grass stunt phytoplasma Kenya
Ngs-phytoplasma-Uganda	Napier Grass Stunt Phytoplasma Uganda
nt	Nucleotide
PCR	Polymerase chain reaction
PGS	Pure germinating seeds
pmol	Pico mol
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
Rpm	Revolutions per minute
rRNA	Ribosomal RNA
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
Tris	Trizma base
tRNA	Transfer RNA
UGA	Uracil Guanine and Adenine
UV	Ultra Violet
vol	Volume
wt	Weight

Abstract

Sampling insects in and around Napier grass fields revealed that 21 Homopteran insect species exploit Napier Grass (*Pennisetum purpureum* Schumach 1827) canopy. Leafhopper *Cicadulina sp.,* and plant hoppers; *Leptodelphax dymas* Young, and *Sogatella manentho* Horvath, are the most dominant Napier canopy insects. This study developed a Loop Mediated Isothermal Amplification of DNA (LAMP) for rapid detection of phytoplasma 16S rDNA. LAMP was simple, sensitive, specific, robust and very rapid Napier Grass Stunt phytoplasma (Ngs-phytoplasma) detection technique. After a 20 days of phytoplasma Acquisition Access Period (AAP) on diseased Napier grass and 30 days latency period on healthy grasses, *Cicadulina sp., Exitianus sp 5*., and *Cofana spectra* Distant tested positive for Ngs-phytoplasma indicating that they interact with Ngs-phytoplasma in circulative manner. The ability of these Napier Canopy insects to vector Ngs-phytoplasma was confirmed by Membrane feeding and Natural transmission experiments, incriminating the white leafhopper, *Cofana spectra* Distant to be the leading insect vector of Ngs-phytoplasma though *Cicadulina sp.,* and *Exitianus sp 5* cannot be ruled out as additional vector(s).

Key Words: Homoptera of Napier grass canopy, Loop mediated isothermal amplification of DNA (LAMP), Napier grass stunt phytoplasma (Ngs-phytoplasma), Transmission experiments.

INTRODUCTION

1.1 Small holder dairy industry in Kenya

Kenya has the most developed smallholder dairy system in sub-Saharan Africa with an estimated dairy herd of 3 million (Smith and Orodho, 2000). The Dairy Sub-sector is the second largest contributor to agricultural Gross Domestic Product after beef, with an estimated 14% of the total agricultural production in 1995 (Orodho, 2006). Milk is the major product from the dairy industry and is produced predominantly by smallholder dairy farmers, in the high agricultural potential areas of Kenya (Mbogo, 1992). The demand for milk in Kenya has continued to rise. It is estimated that current annual milk production level of 2.45 billion litres must be doubled to 5 billion litres to meet the demand by the year 2010 (Muriuki, 1992).

According to Staal *et al.,* (1998), the high potential agricultural areas of Kenya are very densely populated and holding sizes are small; natural grazing is no longer available, so cattle are fed on crop residues, cultivated fodder and some concentrates. McLeod *et al.* (2001) ranked Napier grass fodder the highest (40%) among other fodder types available to cattle in Kiambu (Central Kenya). A survey by Lekasi (2000) showed that farmers commit 21-28% of their land to Napier grass production. Where farms are small, cattle are confined and fed by cut-and-carry, also referred to as zero grazing (Staal *et al.,* 1998). In the last decade, cultivation of Napier grass has boomed in East Africa, as small-scale farmers have shifted to zero grazing. With the expansion of this fodder-crop, however, has come a new disease called "Napier grass stunt (Ngs)" (Farrell, 2002).

1.2 Napier grass

Napier grass (*Pennisetum purpureum* Schumach 1827), or "Elephant grass" is native to Eastern and Central Africa and has been introduced to most tropical and sub-tropical countries (Boonman, 1993). It was named after colonel Napier of Bulawayo in Zimbabwe who championed its adoption as livestock feed in the colonial Rhodesia (Boonman, 1993). In Kenya and Uganda, the grass was introduced as a mulch crop for Coffee during the colonial times, but farmers soon realized and exploited its potential as animal fodder (Orodho, 2006). *Pennisetum* is a genus of grasses in the grass family Poaceae, subfamily Panicoideae, tribe (Paniceae), native to tropical and warm temperate regions of the world. The genus also includes a type of millet, and a pasture form (Kikuyu grass, *Pennisetum clandestinum*) originating from the highlands of Kenya (Boonman, 1993). Napier is a tall grass resembling sugarcane. Mature plants normally reach up to 4m in height and up to 20 nodes. It is a robust perennial forage; with vigorous root system, sometimes stoloniferous with a creeping rhizome (Henderson & Preston, 1977).

It thrives on poorly drained soils to dry sandy soils. However, it grows best in rich well-drained soils. Reported in a several ecological zones ranging from Warm Temperate Dry to Wet through Tropical Dry to Wet Forest Life Zones, The grass is reported to tolerate annual precipitation of 2.0 to 40.0 dm annual temperature of 13.6 to 27.3°C and pH of 4.5 to 8.2 (James, 1983)

Napier grass can out-yield many grasses such as guinea grass (*Panicummaximum*) and Rhodes grass (*Chloris gayana*) (Relwani *et al.,* 1982). It withstands repeated cutting (Purseglove, 1972). Four to six cuts in a year can produce 50-150 tonnes fresh herbage per ha. Its multiple uses include fire breaks, mulch, green manure, wind break, grazing, soil erosion control and as constituent of fish ponds (Farrell *et al.,* 2002). In Kenya, it has been exploited in the novel 'push-pull' cereal stem-borer pest management (Khan *et al.,* 2001). Lekasi, (2000) reported that Napier grass refusals can be used in the cow stalls as bedding to get manure, some farmers use the canes for making chicken houses. Acland (1971) reported the wide usage of Napier grass in Uganda for soil conservation and mulching coffee. Napier Grass is also reported to tolerate drought, fire, frost, fungi, high pH, laterites, low pH, monsoon, savanna, sewage sludge, virus, weeds, and water logging (Duke, 1978). In Spanish Guinea, the leaf and stalk infusion is used as a duiretic in anuria or oliguria. In Central Africa, the leaves are used as a source of a medicinal salt (Watt and Breyer-Brandwijk, 1962).

Napier is a shy breeding grass with seeds having low genetic stability, viability, and very low seed yields - rarely more than 1-2 kg/ha Pure Germinating Seed (PGS) (Van Gastel, 1978). Research efforts to develop seeds were shelved and seed is usually not available to farmers. It is established vegetatively from stem cuttings or crown divisions (Humphreys, 1994). In Kenya many Napier grass varieties have been collected locally, introduced from other African countries or developed by selective breeding (Orodho 2006). The varieties include; Bana grass, French Cameroon, Clone 13, Uganda hairless, Gold Coast, Capricorn, Cubano, Domira, Ghana, Gold Coast, Merker, Merkeron, Mineiro, Napier, Pungwe, Uganda, Urukwanu, Pusa Giant Napier and Mott Napier cultivars (Hann & Monson, 1988). Other varieties include ILRI

Napier accession No. 1671 and 16. Napier grass (*Pennisetum purpureum* (2n=28) can form a hybrid with Bulrush millet (*Pennisetum americanum* (2n=14). A Pakistani Napier hybrid, sometimes called Bajra Napier hybrid is a cross between Napier grass and Bulrush millet. Bana grass was formerly thought to be a hybrid with (2n=21), but was later confirmed just to be Napier grass cultivar since it has 2n =28 (Orodho 2006). Morphological and agronomic characteristics cannot be used to distinguish all the Napier accessions, therefore confusion of varietal names is common. Several varieties circulate under more than one name unnoticed by those responsible.

1.3 Napier grass stunt disease (Ngs-disease)

1.3.1 Symptoms

The disease is usually visible in re-growth after cutting or grazing (Khan *et al.,* 2001). It causes healthy thick Napier grass leaves to turn thin, yellow and weak. A special indication of the disease is the short internodes, affected shoots are severely stunted (Fig. 1). Often the whole stool is affected with complete loss in yield and eventual death.

Fig. 1 A farmer holding Ngs-diseased plant (DNA, July 27th 2007)

1.3.2 History of Ngs-disease

The disease has been present in Eastern Africa for about 30 years but it has become more noticeable in the last few years. Tiley *et al.* (1969) reported a stunting disease of Napier grass in Uganda and the cause was suspected to be a virus transmitted by insects. In Kenya, the disease was first observed in the year 2000 in Bungoma (Western Kenya) (Khan *et al.* 2001). Two years later, Jones *et al.* (2006) reported a disease with similar symptoms in Ethiopia, meaning that the disease has gained a regional epidemiology.

1.3.3 Etiology of Ngs-disease

Jones *et al.* (2004) identified the cause of Ngs-disease in Kenya and Uganda to be a phytoplasma. Analysis of rDNA sequences [GeneBank accession numbers AY377874-AY37787) identified these phytoplasmas as members of the 16SrXI (rice yellow dwarf, Candidatus Phytoplasma oryzae) group (Fig. 3). Two years later, 16S rDNA of Napier grass stunt phytolasma in Ethiopia was sequenced and its phylogeny determined by blast analysis. The nucleotide sequence deposited in GenBank (Accession No. DQ305977) showed that these phytoplasmas are similar to the African sugarcane yellow leaf phytoplasma (Accession No. AF056095), a member of the 16SrIII group; Candidatus Phytoplasma pruni (Fig. 3) (Jones *et al.,* 2006).

1.3.4 Identifying the insect vector of Ngs-phytoplasma

Napier Grass Stunt Disease (Ngs-disease) is most severe in Bungoma (Western Kenya), where natural transmission is believed to have occurred (Khan personal communication). Since there is no parasitic plant present where Napier grass is grown, either the plants arrived as infected seedlings and/or insects carrying the phytoplasma infected them. Phytoplasmas are phloem limited, therefore are transmitted by phloem feeding plant and leafhoppers (Phyllis *et al.,* 2006). The putative insect vector(s) of NGS-phytoplasma are still unknown. Information is thus needed on potential vectors and inoculum sources to predict the risk of new infections (in one or multiple crops), to monitor disease progress, and to develop control methods.

1.4 Phytoplasmas

'Phytoplasma' is a trivial name that collectively refer to wall-less, insect transmitted, non-helical prokaryotes that were formerly known as mycoplasma-like organisms (MLOs) (Razin *et al.*, 1998). Together with mycoplasmas and spiroplasmas, they comprise the smallest and simplest self-replicating cellular organisms, belonging to the class Mollicutes ("molli" - soft; "cute" – skin). Mollicutes represent a branch of the phylogenetic tree of the gram-positive eubacteria and are most closely related to low-GC gram-positive bacteria such as *Bacillus*, *Clostridium*, and *Streptococcus spp*. (Weisburg *et al.,* 1989).

1.4.1 Phytoplasma ecology

Phyllis *et al.* (2006) noted that both insects and plants are natural hosts of phytoplasmas (Fig. 5). They are unique bacteria, as they can efficiently invade cells of insects and plants, organisms belonging to two kingdoms. They inhabit the phloem sieve elements of infected plants and the gut, haemolymph, salivary gland and other organs of sap-sucking insect vectors (Kirkpatrick, 1991).

1.4.2 Phytoplasma genetics

Phytoplasmas have small genomes (108 -109 daltons) with low G+C content (23 – 41%), AT-rich, few rRNA operons and few tRNA genes (Kollar and Seemüller, 1989). Whereas most mollicutes use UGA as a tryptophan codon instead of a stop codon, a feature they share with mitochondria, the phytoplasmas retained UGA as a stop codon (Razin *et al.,* 1998). Many genes coding for essential metabolic pathways in other organisms are missing in phytoplasmas (Bai *et al.,* 2006). The small genome in phytoplasmas is due to reductive evolution from Bacillus/Clostridium ancestors. They have lost 75% or more of their original genes and can no longer survive outside insects and plant hosts. This is why they have not been cultured in cell-free medium (Firrao *et al.,* 2005).

Many phytoplasmas contain two rRNA operons (Fig. 2); 16S and 23S (the S in 16S represents Svedberg units) (Kuske and Kirkpatrick, 1992). They have a tRNA gene in the spacer region between the 16S and the 23S (Smart *et al*., 1996). Sub-unit 16S is synthesized from a sequence of nearly 1,500 nucleotides. Sub-unit 23S is synthesized from a sequence of nearly

3,000 nucleotides. rRNA genes are largely used to detect polymorphism among bacteria because they represent about 0.1% of the genome and are highly conserved. For this reason, genes that encode the rRNA (rDNA) are sequenced to identify an organism's taxonomic group, calculate related groups, and estimate rates of species divergence. Currently, the classification of phytoplasmas is based on the nucleotide sequence of the 16S rRNA gene. 16S rRNA gene sequence, most studied among prokaryotes, is a major molecular characteristic for Phytoplasma definition and identification (Gundersen *et al*., 1994).

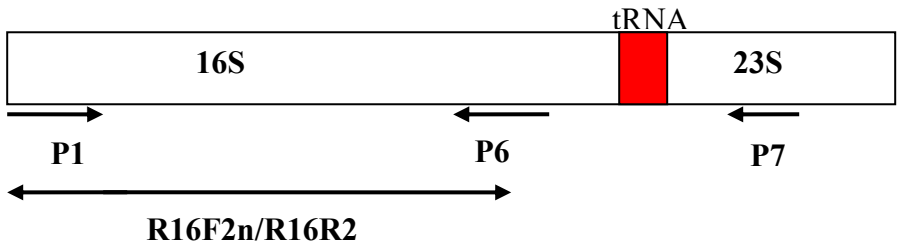


Fig. 2 Structure of rRNA operon showing the 16S, tRNA spacer region and 23S ribosomal units. Regions amplified in a Nested PCR using universal primer sets P1/P6-R16F2n/R16R2 are also shown. {Modeled by the author using information from Maria *et al*., (2001)}

1.4.3 Phytoplasma classification

In 1993, the trivial name phytoplasma was proposed to the International Committee on Systematic Bacteriology (ICSB) Subcommittee on the Taxonomy of *Mollicutes* (Sears and Kirkpatrick, 1994). Recently, phytoplasmas were assigned to a novel genus, "*Candidatus* Phytoplasma" a designation that clearly reflects their plant host association (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group, 2004). Figure 3 shows the major phytoplasma taxonomic groups and the diseases associated with them

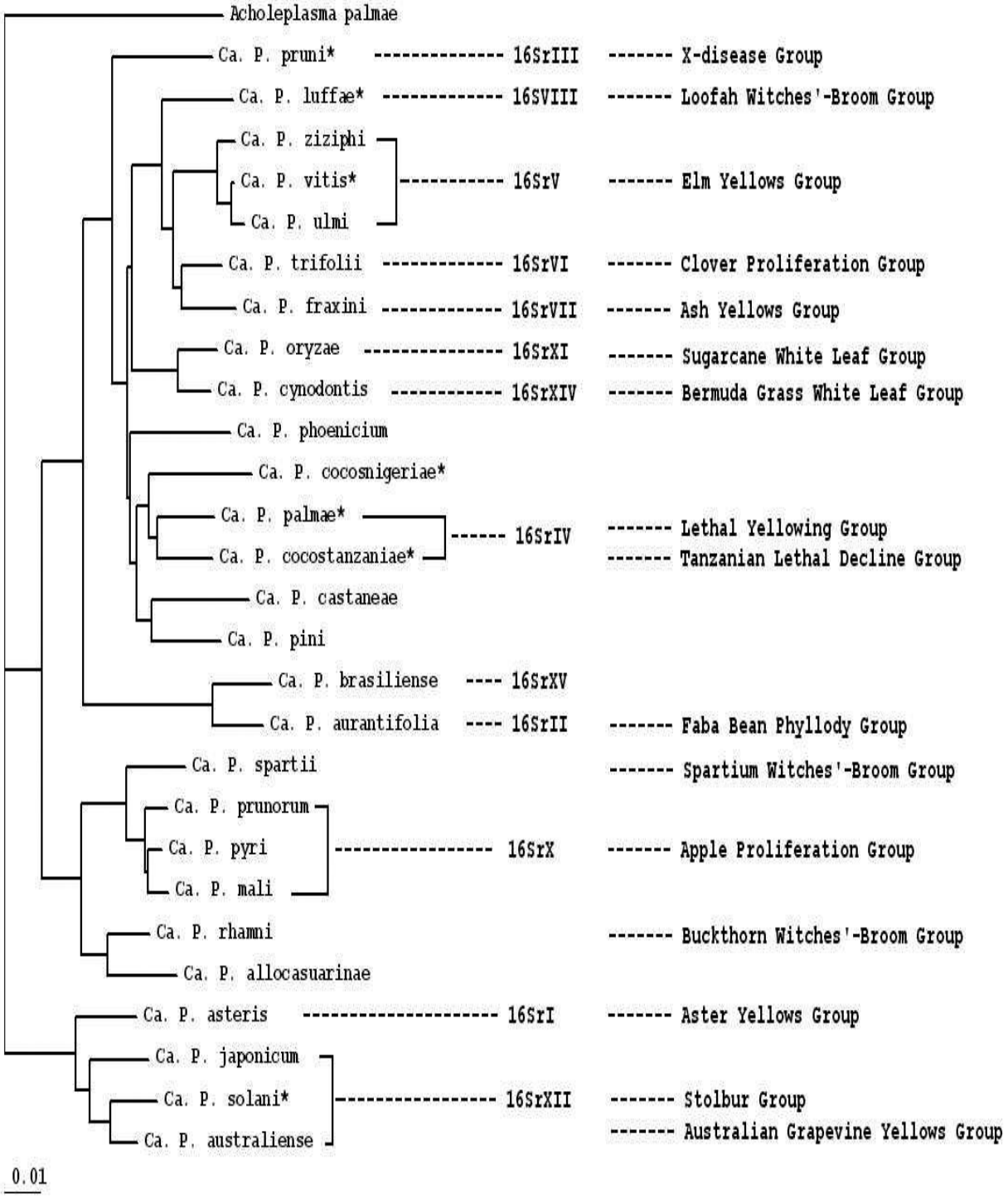


Fig. 3. Phylogenetic tree of the genus '*Candidatus* Phytoplasma' and their relationships. The tree was constructed using CLUSTALX by alignment of *Candidatus* species 16S rDNA sequences, publicly available at TreeBase (http://www.treebase.org/treebase/console.html) as matrix accession M1788 (Thompson *et al.*, 1997).

1.4.4 Pathogenicity of phytoplasma infections

Phytoplasmas cause hundreds of plant diseases, several of which have world-wide agricultural significance (McCoy *et al*., 1989). Phyllody, the production of leaf like structures in place of flowers, suggesting that the phytoplasma downregulates a gene involved in petal formation and genes involved in the maintenance of the apical meristem (Pracrose *et al.,* 2006). This causes sepals to form where petals should. Other symptoms, such as the yellowing of leaves, are thought to be caused by the phytoplasma's presence in the phloem affecting its function, and changing the transport of carbohydrates (Muast *et al.*, 2003). Phytoplasma infected plants may also suffer from virescence - the development of green flowers due to the loss of pigment in the petal cells (Lee *et al.,* 2000). Sometimes sterility of the flowers is also seen.

Many phytoplasma infected plants gain a bushy or witch's broom appearance due to changes in normal growth patterns caused by the infection. Most plants show apical dominance but phytoplasma infection can cause the proliferation of auxiliary (side) shoots and an increase in size of the internodes (Lee *et al.,* 2000). Such symptoms are actually useful in the commercial production of Poinsettia. The infection is necessary to produce more axillary shoots that enable to production of pionsettia plants that have more than one flower (Lee *et al.,* 1997).

Phytoplasmas may cause many other symptoms that are induced because of the stress placed on the plant by infection rather than specific pathogenicity of the phytoplasma. Photosynthesis, especially photosystem II, is inhibited in many phytoplasma infected plants (Bertamini *et al.,* 2004). Phytoplasma infected plants often show yellowing which is caused by the breakdown of chlorophyll, whose biosynthesis is also inhibited (Bertamini *et al.,* 2004). In addition,

phytoplasmas import numerous metabolites from the host plant, which eventually could change the host physiological equilibrium.

1.4.5 Detection of phytoplasma

Microscopic techniques have been tried to detect phytoplasmas but with little success (Chen *et al.,* 1989). Other bacteria present in the phloem can interfere. It can be difficult to identify true phloem cells in the microscope and plant material (present in cells other than the phloem).

Because phytoplasmas are unculturable, numerous molecular techniques have been developed and applied to phytoplasma research; foremost among them is PCR (Lee *et al.,* 2000). Based on the conservative nature of ribosomal DNA across all prokaryotic organisms, total DNA from plants or insects is used as a template for short synthetic primers. Universal primers amplify sequences (Table 1) common to all phytoplasmas and can be used to determine if phytoplasma DNA is present (Lee *et al.,* 1993). Specific primers amplify some of the variable regions and have been developed for most of the phytoplasma groups (Lorenz *et al.,* 1995).

Table 1 Generic primers used in selective amplification of phytoplasma rRNA operon.

Primers	Specificity	Expected base pairs	Developed by
P1/P7	universal phytoplasma	~1800	Deng and Hiruki 1991
P1/P6	Universal phytoplasma	~ 1500	Deng and Hiruki, 1991
R16F2n/R16R2	universal phytoplasma	~1250	Lee *et al.,* 1993

To increase specificity, nested PCR, in which amplification by one primer is followed by amplification with a second more specific primer or one that amplifies a smaller sequence within the first product, has been developed to multiplex nested PCR, in which multiple primers are used (Daire *et al.,* 1997). Real-time PCR (RT-PCR) can be used to quantify phytoplasma units in a reaction mixture. It has so far been employed to quantify the movement and multiplication of phytoplasma in plants (Christensen *et al.,* 2004) and could be applied to insect vectors. Webb *et al.,* (1999) developed *in situ* PCR (on fixed and sectioned plants and on whole insects) using 20- to 24-mer oligonucleotide primers. Their findings from studies using *in situ* PCR were in strong agreement with previous electron microscopic and immunochemical studies. This technique allows for a more efficient and effective study of the biology and epidemiology of multiple infections in a single host and of the events leading to transovarial transmission.

Recently, several phytoplasma proteins were successfully expressed in *E. coli*, and used as antigens to obtain phytoplasma-specific antibodies. These proteins were unidentified membrane proteins (Blomquist *et al.,* 2001), the SecA protein of the secretion system (Kakizawa *et al.,* 2001), and Rep proteins encoded in extrachromosomal DNA (Nishigawa *et al.,* 2001). These successes were based on the finding that phytoplasmas use the same universal codon system as E. coli and other bacteria for protein synthesis, unlike most other mycoplasmas (Miyata *et al.,* 2002). Microscopic techniques have also been tried to detect phytoplasmas but with little success (Chen *et al.,* 1989). Other bacteria present in the phloem can interfere. It can be difficult to identify true phloem cells in the microscope and plant material (present in cells other than the phloem).

1.4.6 Seeking a reliable phytoplasma detection technique

Plants infected with Napier grass stunt disease (Ngs-disease) appear healthy for up to 1 year (Khan Pers. Comm.). Therefore, many infected planting materials are propagated unnoticed, by the farmer. The need for a simple, rapid, sensitive and specific Ngs-phytoplasma detection technique is long overdue. Detection of phytoplasmas have been largely based on the selective amplification of the 16S phytoplasma rDNA by PCR (Deng and Hiruki, 1991). However, the uneven distribution of phytoplasmas in plant tissue often makes detection unreliable (Gundersen & Lee 1996). Some PCR primers can induce dimers or unspecific bands. They also have sequence homology in the 16S-spacer region to chloroplasts and plastids increasing the risk of false positives. Nevertheless, PCR process is multiple and complex, technically demanding and requires up to 4 hours to complete, making it impractical for rapid monitoring of active phytoplasma infections.

There are several alternative methods for gene amplification such as; nucleic acid sequence-based amplification, self-sustained sequence replication and strand displacement amplification (Norihiro *et al.,* 2008). Notomi *et al.* (2000), while working with HBV viral DNA, reported a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP). LAMP is an autocycling DNA synthesis performed using a DNA polymerase with a high level of strand displacement activity and a set of specially designed inner (FIP and BIP) and outer primers (F3 and B3) known to hybridize with six distinct sequence sites of the template DNA. The cycling reaction continues with accumulation of 10^9 copies of target DNA in less than an hour (Notomi *et al.,* 2000). The final products of LAMP are stem–loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand.

1.4.7 Principle of Loop mediated isothermal amplification of DNA

Figure 4 illustrates the principle of LAMP, six distinct regions are designated on the target DNA, labeled B3, B2, B1, F1c, F2c and F3 from the 5' end. As ‘c’ represents a complementary sequence, the F1c sequence is complementary to the F1 sequence. Two inner primers (FIP and BIP) and outer primers (F3 and B3) are used in the LAMP method. FIP (BIP) is a hybrid primer consisting of the F1c (B1c) sequence and the F2 (B2) sequence. Four primers are simultaneously used to initiate DNA synthesis from the original un-amplified DNA, therefore, target selectivity and specificity is higher than those obtained in PCR (Notomi *et al.,* 2000).

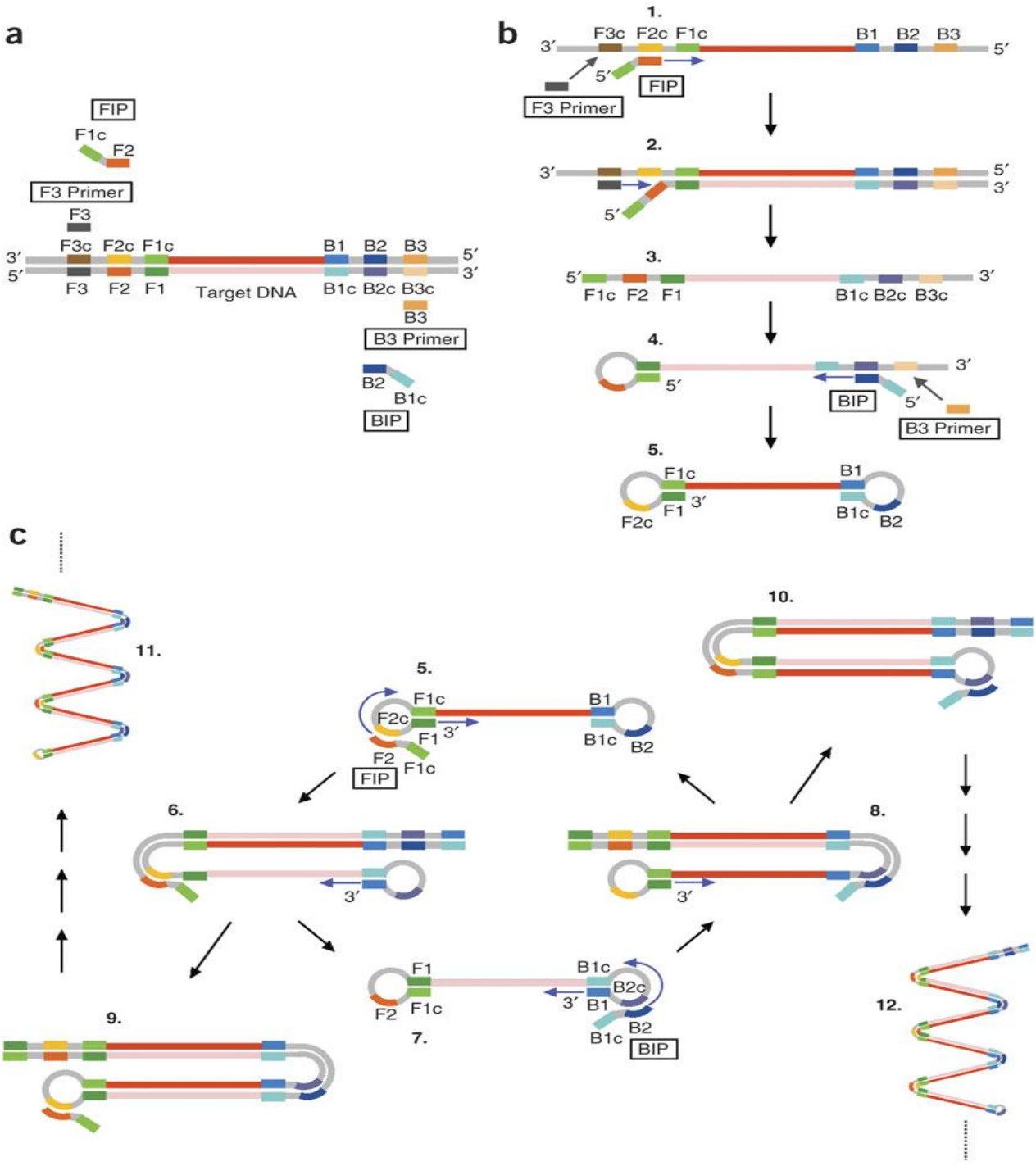


Fig. 4 Model of loop-mediated isothermal amplification (LAMP) process (Norihiro *et al.,* 2008)

1.4.8 Transmission of phytoplasmas

Three known mechanisms introduce phytoplasmas into the vulnerable tissue of host plants: (*a*) vegetative propagation or grafting of infected plant material, (*b*) vascular connections made between infected and noninfected host plants by parasitic plants such as dodder (*Cuscuta* spp.) (Dale and Kim 1969) and (*c*) vector insects feeding on non-infected host plants (Fig. 5) (Kirkpatrick, 1991). Recent reports suggest a fourth possible source of phytoplasma: seed transmission; lethal yellows phytoplasma has been detected in coconut fruit embryos from infected trees (Cordova *et al.,* 2003) and alfalfa witches' broom has been detected in alfalfa seeds from phytoplasma-infected parent plants (Khan *et al.,* 2002). Kawakita *et al.,* (2000), found phytoplasma in the eggs laid on mulberry shoots by inoculative leafhoppers and in first-instar nymphs hatched from these eggs, showing that phytoplasmas can be trans-ovarially transmitted. Figure 4 illustrates a generalized life cycle of phytoplasma

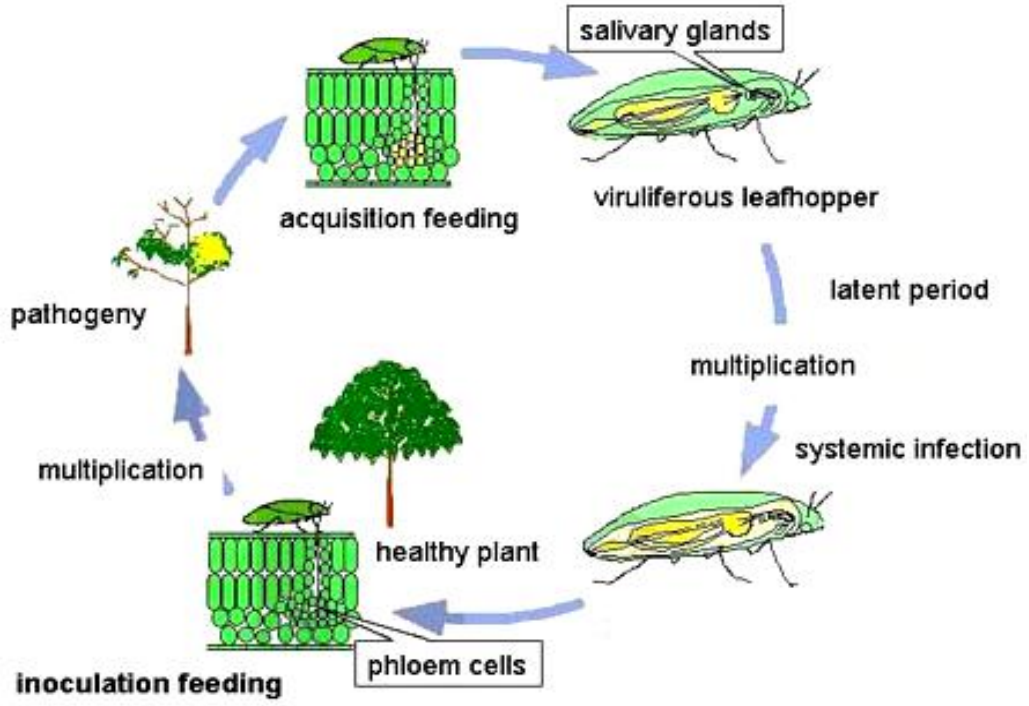


Fig. 5 Generalized Life cycle of phytoplasmas (http://papilio.ab.a.u-tokyo.ac.jp/planpath/phyto-genome/what.htm, 2008).

1.5 Objectives of this study

This is a dual study with two broad objectives; a) To develop a rapid, simple, sensitive and reliable phytoplasma detection technique and b) To determine the putative insect vector of Napier grass stunt phytoplasma (Ngs-phytoplasma).

The specific objectives of this study include:

1. To develop and optimize working conditions of LAMP primers.
2. Evaluate the sensistivity and specificity of LAMP assay.
3. Sample and rear Homoptera (except aphids) found in and around Napier grass fields.
4. Monitor the growth rate of the sampled insects, to estimate their relative abundance in the field.
5. Carryout membrane feeding assay to test the inoculative abilities of adult female insects sampled from Napier canopy, and to isolate pure cultures for future use in Ngs-phytoplasma ELISA.
6. Set up long term Natural transmission assay to test the ability of Napier Canopy insects to inoculate Napier grass with Ngs-phytoplasma.

MATERIALS AND METHODS

2.1 Study area

Bungoma district is located in the western frontier of Kenya (Fig. 6), bordering Uganda. It receives about 1200mm of rainfall per annum, hence classified as a high agricultural potential area (Withaka *et al.,* 2002). Like other high agricultural potential areas of Kenya, such as Central province, and Nyanza, there is rapid population growth, making it densely populated and farm holding sizes very small. Small holder dairy farming is widely practiced as natural grazing is no longer available. Cattle are fed on crop residues, cultivated fodder and some concentrates. This has lead to great intensification of Napier grass farming, and emergence of Napier Grass Stunt Disease (Ngs-disease) (Waithaka *et al.,* 2002).

Even though Ngs-disease is widespread in the larger western province (Fig. 6), Bungoma District is the epidemic zone of Ngs-disease in Kenya (Khan Pers. Comm.), with many Napier fields reduced to stunted yellowish shoots. We therefore selected Bungoma District to; a) Sample Homopteran insects of Napier canopy, b) Collect Napier grasses susceptible to Ngs-phytoplasma for use in the transmission experiments.

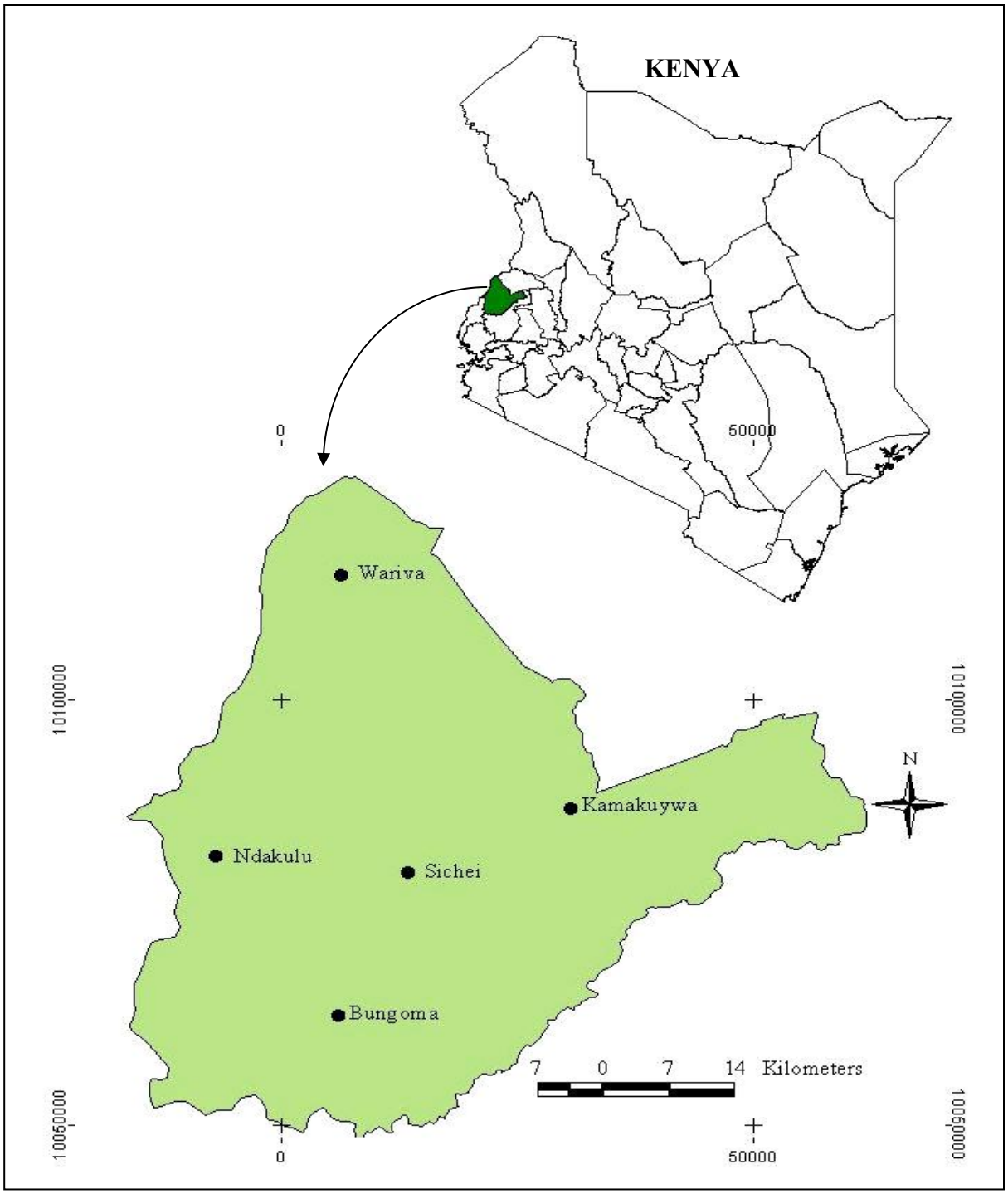


Fig. 6 Map of Bungoma District and its location in Kenya. The dots are areas within Bungoma District where sampling of Napier canopy insects was done. The map was generated using ArcView 3.2 (ESRI, 1996)

2.2 Napier grass used in this study

Napier grass accession susceptible to Ngs-disease obtained from the disease endemic areas (Bungoma-Kenya) was used throughout the study. Both diseased and healthy grasses were maintained in separate sections of insect-free glasshouses at 25–28 ^{0}C and 65–70% RH and their phytoplasma status determined by PCR. Healthy plants were raised from stem cuttings of phytoplasma negative grasses, and were never exposed to disease transmitting insects. Diseased Napier were utilized for acquisition feeding by the candidate insects while healthy Napier were used as the plant hosts during the phytoplasma latency period.

2.3 Homoptera of napier canopy (HNC)

2.3.1 Sampling Homoptera of Napier Canopy.

Field sampling was done by Malaise trapping, sweep netting and in some cases the insects were hand picked by an aspirator. The Malaise trap was positioned in the Napier grass field to monitor movement of insects in and out of Napier grass field. The trapped insects comprising of several species were sorted out. Homopteran insects (except aphids) were grouped according to external morphological characteristics and used as template specimens for compiling data on Homoptera of Napier canopy (HNC). Sweep netting or sucking by an aspirator were used to sample insects alive.

Sampling was done in four locations chosen within Bungoma District; Ndakulu (West), Bungoma (South), Sichei (Central), Kamakuywa (East) and Wariva (North) (Fig. 6). Two

Napier grass plots (Diseased) were selected randomly from each location, and each plot was divided into 5 randomly located transects. Each transect sample consisted of 10 sweeps in a straight line with a 38-cm diameter. Beating - knocking Napier grass was used to agitate resting insects, which would then be trapped in the sweep net. After sweeping, arthropods in the nets were hand picked by aspirator, plant and leafhoppers were sorted morphologically and transferred to ICIPE'S TRO campus (Mbita) rearing units. The sampled insects were provisionally named from 'hopper 1 to hopper 21 depending on how readily and easily they were sampled.

2.3.2 Rearing Homopteran insects sampled from Napier grass

In the glass house, at 25 – 28^{0}C and 65-70% RH, the insects were maintained in cages (60 cm high and 25 cm in radius) made up of wooden frame (Fig. 7). Top and side openings of the cage were covered with fine nylon mesh for aeration. Healthy Napier grass collected from non-diseased plots in Bungoma (western Kenya), were used for rearing, to act as food and breeding ground for the insects. Phytoplasma status of the grasses were ascertained by PCR testing (Data not shown).

Fig. 7 The author, using an aspirator to infest a rearing cage with Homoptera of Napier canopy (HNC) (Picture courtesy of Jimmy Pitchar, ICIPE, 2008).

2.3.3 Taxonomic identification of insects sampled from Napier grass

The insects were taxonomically identified in collaboration with Dr. M. R. Wilson, of the Department of Biodiversity & Systematic Biology, National Museum of Wales, Cardiff, UK

2.3.4 Monitoring growth progress of insects sampled from Napier grass.

The population of each species of Homopteran insect in the rearing cage was sampled at day1 and later at day 60, this data was used to calculate the rate of population increase, according to the differential equation of exponential population growth (Robert, 1993); dN/dt = rN, where rN= intrinsic rate of population increase, dN = change in population, dt = time lapse of the

population change. The rN value obtained was used to estimate the relative abundance of Homopteran insects in the field.

2.4 Development and evaluation of a loop mediated isothermal amplification of DNA (lamp)

2.4.1 Design and optimization of lamp primers

Phytoplasma 16S rRNA gene sequence in the GenBank accession No. AY377874; Napier grass stunt phytoplasma, Kenya was used as the target sequence to design the LAMP primers. Six distinct regions (B1–B2-B3 and F1–F2-F3) were designated on the targeted, then a set of 4 primers, outer (Phyto-F3 and Phyto-B3) and two inner (Phyto-FIP and Phyto-BIP) primers, capable of recognizing the six regions on the targeted DNA, were designed by the author and synthesized by MWG Oligo-synthesis.

2.4.2 DNA extraction

Prior to the LAMP assay on Napier grass phytoplasma (Ngs-phytoplasma) rDNA, leafcuts of Healthy and Ngs-diseased grasses were obtained from *icipe's* TRO Campus (Mbita point field station) in western Kenya. DNA was extracted according to Doyle and Doyle (1990) with slight modification. Four grams of Napier grass leaf tissue was frozen in liquid nitrogen and ground to a fine powder in a 1.5-ml microcentrifuge tube by a homogenizer. 800 µl of Cetyltrimethylammonium bromide (CTAB) buffer was added at 60°C (CTAB buffer: 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0, 0.2% 2-mercaptoethanol). The slurry was incubated in a 60°C water bath for 30 minutes, and then cooled on ice for 5 minutes. The sample was then mixed with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 4000 rpm for 15 min. The supernatant was transferred to a new tube

and 600 µl of cold isopropanol was added and gently mixed. Total nucleic acids were precipitated overnight at -20° C, then pelleted by immediate centrifugation at 14 000 rpm for 8 min. The pellet was rinsed in 70% ethanol, dried, and re-suspended in 50µl of sterile de-ionized water and stored in a –20°C. The quantity and quality of the isolated DNA was estimated by electrophoresis on Ethidium bromide stained, 0.8% agarose gel.

2.4.3 Loop mediated isothermal amplification of DNA (lamp)

The protocol for LAMP was performed as described by Notomi *et al.* (2000) with slight modification. The LAMP technique was performed in a 25 µl reactionThe reaction mixture contained 40 pmol of each of the primers Phyto-BIP and Phyto-FIP, 5 pmol of the primers Phyto-F3 and Phyto-B3, 1x Bst polymerase buffer (New England Biolabs), 2.5 mM of each dNTP (Genescript corporation), 50ng of target DNA, and 8M Betaine (Sigma Aldrich). The reaction mixture was adjusted to 25 µl using sterile distilled water. The mixture was heated at 95^0C for 5 minutes then chilled on ice, 8U of Bst DNA polymerase large fragment (New England Biolabs) was added followed by incubation at 63^0C for 60 minutes. The reaction was terminated by heating at 80^0C for 10 minutes. DNA from healthy Napier grass leaf tissue was used as a negative control.

2.4.4 Examining the specificity of lamp

The smallest band size in LAMP can be predicted, and used in examining specificity of LAMP while the largest band size is unpredictable; it depends on the efficiency of the cycling process. In our case, structure 2 (Fig 7), which is about 223 bp {(182 bp (targeted size) + 41 bp (FIP nucleotides)}, with Phyto-FIP primer attached, was the smallest expected band size produced. Specificity of LAMP was evaluating the success of a negatively controlled LAMP process.

2.4.5 Examining the sensitivity of lamp

The sensitivity of LAMP assay was determined by amplifying 4 $x10^{-1}$ serial dilutions of the DNA target. Phytoplasma detection by PCR was carried out to compare the levels of sensitivity.

Two "universal" phytoplasma-specific nested primer sets; P1/P6 and R16F2n/R16R2 to amplify 1.2 kb of the portion of 16S rDNA, were used to detect Ngs-phytoplasma by PCR. Reaction volumes each containing 50ng of DNA template, 50ng of each primer, 125µM of each dNTP, 1 U of Taq DNA polymerase (Genescript corporation), 1x of standard PCR buffer with 1.5 Mm $MgCl_2$ (Genescript corporation). The reaction mixture was adjusted to 25 µl by sterile distilled water. PCR was performed for 35 cycles in a PTC-100 programmable thermal controller (MJ Research, Inc.) with hot bonnet. Reaction conditions were as follows: 2 min at 94°C, 1 cycle; 1 min at 94°C, 2 min at 52°C (55°C for R16F2n/R16R2), 3 min at 72°C, 35

cycles; and 10 min at 72°C, 1 cycle. Negative control included healthy Napier grass leaf tissue.

2.4.6 Detection of PCR and lamp products

After PCR and LAMP, 8µl aliquots of the products were detected by gel electrophoresis on Ethidium bromide stained 1% Agarose gel using 1x TAE (40 Mm Tris-acetate, 1 Mm EDTA) as running buffer. DNA fragments were visualized by UV trans-illumination, and photographed.

2.4.7 Molecular typing of Lamp products

To confirm the structure, 10 µl of the LAMP amplified products were digested with *Rsa*I endonuclease (New England Biolabs) according to the manufactures instructions, and the sizes analyzed by gel electrophoresis on a 1% agarose gel, stained with Ethidium bromide, using 1x TAE as the running buffer. The DNA fragments on the gel were visualized by UV trans-illumination, and photographed.

2.5 Acquisition and multiplication of Ngs-phytoplasma in the Napier canopy insects

2.5.1 Process of acquisition feeding

To obtain inoculative insects, 4th instar nymphs were caged with diseased Napier for an acquisition access period (AAP) of 20-days under green house conditions. After the AAP, five individual insects from each species were sampled and total DNA extracted while other surviving insects were transferred to healthy Napier plants and kept there as long as they survived to allow the phytoplasma multiplication. After 30 days on healthy Napier, 5 surviving insects from each species were sampled and their total DNA extracted. Ten species of Caged NCH insects were used in this experiment as summarized in the table below. To avoid differences due to insect gender, only females were used. Non-inoculated leafhoppers were used as control.

The list of Napier canopy insects used in the study of Ngs-phytoplasma acquisition and multiplication by insects sampled from Napier canopy is shown below.

1. *Cofana spectra*
2. *Rhinottetix sp.*
3. *Glossocratus afzalii*
4. *Thriambus levus*
5. *Sogatella manentho*
6. *Cicadulina sp.*
7. *Thriambus strenuous*
8. *Leptodelphax dymas*
9. *Exitianus sp. 5*
10. *Recilia banda*

2.5.2 Extraction of DNA from the insect tissue, PCR and Lamp

The original method of Maixner *et al.* (1995) was modified to extract DNA from the insect tissue. Each insect was ground in extraction buffer (100 mM Tris-HCl at pH 8.0, 2% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 20 mM EDTA, and 0.2% 2-mercaptoethanol) at a ratio of 1:5 (wt/vol, tissue/buffer). The slurry was incubated for 30 min in a 60°C water bath, cooled on ice for 5 min and centrifuged at 3000 rpm for 10 min. The supernatant was carefully drained and extracted with 1 volume of chloroform/isoamyl alcohol (24:1, vol/vol). Following 30 minute incubation at –20°C, the DNA was then pelleted at 12,000 rpm for 30 min. The pellet was washed twice with 70% ethanol and re-suspended in 50 µl of 10 mM Tris and 1 mM EDTA at pH 8.0 (TE).

To obtain DNA for molecular analysis, 5 µl of DNA extracted from insects of the same species were pooled (5 x 5 = 25 µl each). Phytoplasma status of each pool was determined first by One-round PCR, then Nested PCR and finally by LAMP (Described in 2.3), 5 trials each. To obtain the phytoplasma infection rate after 30 days on healthy grasses, phytoplasma status of individual insects from the positive pools were analyzed by LAMP technique.

2.6 Membrane feeding experiment

2.6.1 Acquisition feeding

The list below shows the Napier canopy insects used in the Membrane feeding experiment

1. *Recilia banda*
2. *Exitianus sp. 5*
3. *Cicadulina sp.*
4. *Cofana spectra*
5. *Thriambus levus*
6. *Cofana unimaculata*
7. *Thriambus strenuous*
8. *Rhinotettix sp.*
9. *Sogatella manentho*
10. *Leptodelphax dymas*
11. *Glossocratus afzalii*
12. *Cofana polaris*

Twenty, fourth-instar nymphs, of each insect species, were obtained from the healthy colonies in cages and reared on diseased Napier grass for15 days to acquire Ngs-phytoplasma (acquisition feeding). To confirm acquisition, the insects were characterized as carrying the Ngs-phytoplasma by PCR analysis (data not shown). They were subsequently transferred to healthy plants for 30 days to allow the phytoplasma to multiply and the insects to become inoculative prior to the membrane feeding experiment. During membrane feeding, the insects feed by probing the parafilm and sucking the diet in the same way in which they normally probe plant tissues and suck phloem sap. DNA was individually extracted from each feeding medium ration and analyzed by PCR for the presence of NGS-phytoplasma.

2.6.2 The process of membrane feeding

Original method of Zhang *et al.,* (1998) was used. five female NCH (from each species) from the inoculated culture were used in this experiment. White micro centrifuge tubes (1.5 ml) were used as insect chambers. Their cylindrical cups were filled with 200 µl of 5% sucrose in TE (10 mM Tris (pH 8.0), 1 mM EDTA) and sealed with Parafilm and tightened. The bottom ends of the micro centrifuge tubes were cut, an individual insect was placed in each, and the cut end was sealed with cotton wool. Each tube, containing an individual leafhopper, was kept at 23 to 25°C as long as the hoppers lived in a horizontal position with the cap facing a light source to attract the insects to the feeding medium. The parafilm was tightened to simulate the normal phloem pressure in the plant. To curb variations due to gender, only female insects were used.

2.6.3 Isolation of phytoplasma rDNA from the sucrose-TE ration.

Phytoplasma particles were pelleted out of the feeding solution by centrifugation at 12,000 × g for 15 min, 10 µl of 0.5 M NaOH was added and vortexed gently, followed by addition of 20 µl of 1 M Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate (sds) and 20 mM EDTA. Total nucleic acids was extracted with equal volume of chloroform:isoamyl alcohol (24:1), precipitated overnight at –20 with 2 volumes of ethanol, then pelleted by immediate centrifugation at 14 000 rpm for 8 min. The pellet was rinsed in 70% ethanol, dried, and re-suspended in 50µl of sterile water and stored at –20°C.

To obtain DNA for molecular analysis, similar DNAs (from Sucrose-TE of same insect species) were pooled by aliquoting 5µl of each into one tube to make a 25µl template for use

in PCR. The pooled DNA were allowed to stand overnight, vortexed gently and used as template in a nested PCR reaction (Already described in 2.3).

To determine the transmission rate of each insect, the individual DNA samples from the positive pools were subjected to PCR analysis. After 5 PCR trials, pools where no specific amplification was observed were considered phytoplasma negative, no inoculation had taken place, and the insects were exonerated as possible phytoplasma vectors.

2.6.4 Molecular typing of phytoplasma rDNA from the sucrose-TE ration.

Amplicons from the Nested PCR were used as a template in a single restriction digest with the restriction enzymes *Alu*1 and *Rsa*1 (New England Biolabs) according to manufactures instructions. After restriction digestion, 8µl of the Nested PCR product and 10µl of the restriction digests were electrophoresed through 1% agarose gel using TAE (40 Mm Tris-acetate, 1 Mm EDTA) as running buffer. DNA fragments were stained with Ethidium bromide (EtBr), visualized by UV trans-illumination, and photographed.

The RFLP patterns (the sum result of 2 enzymes) of the 16S rDNA gene, Ngs-phytoplasma, Kenya, were compared with the Ngs-phytoplasma, Kenya *Rsa*1 site map of Arocha *et al.,* (2007) (Unpublished data) and analyzed by the method of Nei & Li (Lydia *et al.,* 2007). The similarity coefficient (*F*) of strains *x* (Actual *Rsa*1 digestion) and *y* (*Rsa*1 restriction map of Arocha *et al.,* 2007) were calculated as $F = 2Nxy/(Nx+Ny)$, in which *Nx* and *Ny* are the number of fragments resulting from digestions by enzymes in strains *x* and *y*, respectively, and *Nxy* is the number of fragments shared by the two strains. Genetic distance (D) between strain 'x' and 'y' was calculated as 1-F.

2.7 Natural transmission experiment

2.7.1 Test plants for natural transmission experiment

Napier grass stunt disease (Ngs-disease) symptomatic and assymptomatic plants were collected from Bungoma (Western Kenya). These plants were provisionally identified by foliar symptoms. Each plant was carefully removed from the ground, removing the soil surrounding the bulk of each plant's root system. Leaf cuts from symptomatic and assymptomatic plants were taken and their phytoplasma status determined first by PCR and later by LAMP (data not shown). To obtain plants for the transmission experiments, each plant was transferred to a 5L pot filled with a simple potting mix (Fig. 8) and watered regularly. When preparing plant samples for the Natural transmission experiment, the foliage was rinsed thoroughly with water prior to introduction of the test insect to ensure that all other insect species were removed. Table 2 shows the Napier canopy insect species used in the natural transmission assay, and how many times the treatment was replicated.

Fig. 8 Setup for Natural transmission experiment. Foreground; two potted healthy plants. Background; a potted diseased plant. (Picture courtesy of Jimmy Pitchar, ICIPE, 2008)

Table 2. Napier canopy insects used in the Natural transmission experiment, and the number of plants replicated for each insect.

	Insects	n
1.	*Cofana spectra*	61
2.	*Extianus sp. 5*	30
3.	*Cicadulina sp.*	37
4.	*Leptodelphax dymas*	39
5.	*Sogatella manentho*	33
6.	*Thriambus levus*	36
7.	*Thriambus strenuous*	21
8.	*Glossocratus afzelii*	15
9.	*Recilia banda*	12
10.	*Rhinotettix sp.*	19

2.7.2 Natural transmission tests

Both inoculation feeding on diseased grass, latency and pathogen transmission to healthy grasses were performed in a single insect rearing cage (Fig. 8). In the glass house, at 25–28 ^{0}C and 65–70% RH, the insects were captured by an aspirator and sorted into species. A total of 10 individuals (7 females and 3 males) of each species were placed into a cage of potted diseased Napier. A pot with healthy Napier was also introduced into the same cage. After 90 days, leaf-cuts of the Exposed plants were sampled for DNA isolation and phytoplasma detection. The Exposed plants were then quarantined until the appearance of well-developed symptoms. Unexposed healthy plants were used as control setup.

2.7.3 DNA extraction and lamp

DNA from the sampled leaves of Exposed plants were extracted according to the method of Doyle and Doyle (1990) (already described in 2.3 above) prior to the Loop Mediated Isothermal Amplification of DNA (LAMP).

To obtain DNA for molecular analysis, DNA extracted from grasses exposed to one insect species, were pooled by aliquoting 5µl of each DNA into a sterilized 1.5ml microcentrifuge tube. The pooled DNA was allowed to stand overnight , vortexed gently prior to LAMP. LAMP was performed according to the method of Notomi *et al.,* (2000) (already described in 2.3 above), followed by separation of the products on a 1.5% agarose gel stained with Ethidium bromide. DNA were observed under UV trans-illuminator and photographed.

After 5 LAMP trials, pools not showing DNA bands were considered phytoplasma negative and their corresponding insects exonerated. To evaluate the transmission rate of each insect species, individual plants from LAMP positive pools were screened, and percentage transmission calculated.

RESULTS AND DISCUSSION

3.1 Homoptera of Napier canopy (HNC)

Table 3 is a summary of the insects obtained after conducting the field survey to collect Homopteran insect species found in and around Napier grass fields

Table 3. Homopteran insects (Except Aphids) sampled from Napier grass canopy

Hopper	Nomenclature	Family
1a	*Cofana unimaculata* (Signoret)	Cicadellidae
1b	*Cofana spectra* (Young)	Cicadellidae
1c	*Cofana polaris* (Distant)	Cicadellidae
2	*Poophilus sp.*	Aphophoridae
3	*'Clovia' sp.*	Aphophoridae
4	*Extianus sp. 4*	Cicadellidae (Chiasmini)
5	*Extianus sp. 5*	Cicadellidae (Chiasmini)
6	*Cicadulina sp.*	Cicadellidae (Macrostelini)
10a	*Leptodelphax dymas*	Delphacidae
10b	*Sogatella manentho*	Delphacidae
11a	*Thriambus levus* (Van stalle)	Delphacidae
11b	*Thriambus strenuous* (Van stalle)	Delphacidae
12	*Megalopa sahlbergorum* (Lindberg)	Cicadellidae:Ulopinae
13	*Glossocratus afzelii* (Stal)	Cicadellidae:Hecalinae
14	*Hecalus sp.*	Cicadellidae:Hecalinae
15	*Diostrombus sp.*	Derbidae
16	*Kamendaka sp.*	Derbidae
17	*Nasia nervosa*	Meenophidae
18	*Elasmoscelis sp.*	Lophopidae
19	*Afrosus sp.*	-
20	*Recilia banda* (Kramer)	Cicadellidae:Deltocephalinae
21	*Rhinotettix sp.*	Delphacidae

A total of 21 morphologically different homopteran insects (except Aphids) were found to be associated with Napier grass (Table 3). Five species of delphacidae (planthoppers), Ten species of cicadellidae (Leafhoppers), two species of A Aphrodidae, two species of Derbidae, one species of Meenophidae and one species of Lophopidae. This result indicate that Napier grass support quite a large diversity of homopteran species of insects.

More than 75% of all confirmed phytoplasma vector species are found in the subfamiliy Deltocephalinae (leafhoppers) (Dietrich *et al.,* 2001). Ten species of leafhoppers were sampled from Napier grass field, forming about 48% of Napier canopy Homoptera (Table 3). It is not unusual that all or one of the sampled leafhoppers will vector Ngs-phytoplasma. Aphrodinae (a less derived cicadellid subfamily) are confirmed vectors of phytoplasma (Dietrich *et al.,* 2001), therefore sampling of two Aphrodid species; *'Clovia' sp,* and *Poophilus sp.,* is a good start in Ngs-phytoplasma vector research

Four families of fulgorids: Cixiidae, Delphacidae, Derbidae, and one species in the Flatidae have been confirmed vectors of phytoplasma (Dietrich *et al.,* 2001). The first three families all have at least one species that transmits a phytoplasma in the coconut lethal yellows group (16SrIV). Several species in these families also transmit phytoplasmas from the stolbur (Sr16XII) group. Therefore sampling 5 species of Delphacidae (Table 3); *Leptodelphax dymas Sogatella manentho, Thriambus levus, Thriambus strenuous,* and *Rhinotettix sp.,* and two species of Debridae; *Kamendaka sp. Diostrombus sp.,* shows a good beginning in Ngs-phytoplasma vector research.

No phytoplasma vector research has incriminated the families Meenophidae and Lophopidae as vectors of phytoplasma. This does not mean that they are incapable of vectoring the pathogen. In the Ngs-phytoplasma vector research, their capacity to vector the pathogen will be evaluated.

Cicadellid leafhopper *Cofana spp.,* were the most readily and easily sampled Homoptera of Napier Canopy (Table 3). They were provisionally named hopper #1a (*Cofana polaris*), 1b (*Cofana spectra*) and 1c (*Cofana unimaculata*). *Leptodelphax dymas*, (#10a) and *sogatella manentho,* (10b) were the most easily sampled plant hoppers in the Napier grass fields. The insects were named #10a and #10b because they could not be easily separated morphologically by Mike Wilson and the author identified them as two separate delphacid species in the family Delphacidae.

During the collection the insects were found to exploit different layers of Napier grass canopy, therefore they were collected differentially. Leafhoppers were found to be abundant on the upper Napier grass canopy and were reliably sampled using sweep nets. On the other hand, plant hoppers concentrated more on the lower Napier canopy and were less likely to be caught by sweeping net; they were often picked by an aspirator directly from the plant. Other Napier Canopy Homopteran families were found not to exploit any specific habitat within the Napier canopy. Nevertheless, Malaise traps collected few Homoptera of Napier canopy, more so planthoppers (data not shown).

The abundance of leafhoppers on the upper Napier grass canopy conforms to the general behavior of leafhoppers to be more often located on the leaves of the host plants. Plant hoppers on the other hand exploit any part of the host plant, but mostly the stem and branches, making them less likely to be sampled by sweep netting. This may partly account for why the first 3 insects sampled were leafhoppers or why they were the most abundant Homopteran insects in the Napier canopy

In spite of most hoppers species ability to fly (winged), only very few hoppers were intercepted in Malaise traps (data not shown), showing that hoppers are slow dispersers. Plant hoppers were not easily intercepted by Malaise traps and sweep nets because they tend to concentrate more on the lower sections of the Napier grass hence traps more than 1 m above the ground collected few of them. Otherwise, upper Napier canopy hoppers (Mainly leafhoppers) were more easily caught by flight intercepting devices such as sweep nets and Malaise traps.

3.1.1 Rearing Napier canopy insects

Intrinsic rate of population increase (r) is how much a population can grow between successive time periods (Robert, 1993). Values above zero indicate that the population is increasing. The higher the value of 'r', the faster the intrinsic rate of population growth.

When the insects were reared (in captivity) on healthy Napier grass and their intrinsic rate of multiplication evaluated, *Cofana spectra* (r = 1.63), *Exitianus sp 5* (r = 2.25), *Thriambus levus* (r = 2.45), *Sogatella manentho* (r = 2.33), *Leptodelphax dymas* (r = 2.62)., *Thriambus strenuous* (r = 3.37), *Recilia banda* (r = 1.37), and *Rhinottetix sp*. (r = 3.70) had modest

intrinsic rate of growth on Napier grass (Table 4). The ability of these insects to proliferate on Napier shows that they probe its vascular tissue for food, and therefore can transmit vascular colonizing prokaryotes such as phytoplasma. *Cicadulina sp*., posed robust population growth rate (r = 7.42) on Napier grass (Table 4, Fig. 9), hitting a population of about 500 individuals just in 60 days (Table 4). One reason for this could be their small size and therefore shorter generation time compared to other insects sampled from Napier grass. However, the generous population growth rate could be attributed to their use of Napier grass as a principal host for food, breeding ground and shelter from natural enemies, a characteristic which can make them vector NGS-disease to the outbreak level as witnessed in Bungoma (Western Kenya).

Table 4. Homoptera of Napier Canopy, their population size and rate of population increase (r) when reared in captivity from day 1 to day 60.

Hopper	Days		Survival/Growth rate (r)
	N at Day 1	N at day 60	
Cofana unimaculata	40	50	0.17
Cofana spectra	25	123	1.63
Cofana polaris	28	0	-0.47
Extianus sp. 4	55	0	-0.92
Extianus sp. 5	38	173	2.25
Cicadulina sp.	55	500	7.42
Leptodelphax dymas	43	200	2.62
Sogatella manentho	93	233	2.33
Thriambus levus	28	175	2.45
Thriambus strenuous	63	265	3.37
Recilia banda	33	115	1.37
Rhinotettix sp.	23	245	3.70
Glossocratus afzali	10	20	0.17

Where; N = Population size

R = dN/dt

Napier canopy insects with significantly lower 'r' were *Cofana unimaculata* (r = 0.17) and *Glossocratus afzalii* (r = 0.17) (Table 4). Being relatively larger hoppers, this slow population increase rate was anticipated. We can also hypothesize this low 'r' value to them not preferring Napier grass as a host, making them unable to transmit phytoplasma to the plague status seen in Western Kenya. However, the population growth rate was positive, meaning that they probe the plant vascular tissues for the sugar rich photo-assimilates, hence being able to transmit phytoplasmas.

Cofana polaris and *Exitianus sp 4*, showed negative growth rate on Napier at ICIPE'S TRO campus (Mbita) and most populations collapsed in captivity. However, they were shown to proliferate (but rather slowly) at the Bungoma (Western Kenya) rearing unit. They may have narrow tolerance to changes in environmental conditions and rarely survive outside the field conditions different from that of the area they were sampled from. If incriminated as vector(s), they will restrict NGS-disease epidemic to the Bungoma area (where they were sampled), leaving other areas with different environmental conditions safer. Nevertheless, these insects may not prefer Napier grass as a host. It is unlikely that they can vector NGS-phytoplasma to an outbreak scale as witnessed in Bungoma (Western Kenya).

Earlier reports indicated that *Diostrombus sp., Kamendaka sp.,* and *Nasia nervosa* were not breeding on Napier grass (ICIPE phytoplasma working team) and therefore are less likely to be the vector(s) of NGS-phytoplasma. However, according to Power (2000), a vector does not

need to breed on the diseased plant, or prefer to feed on it, to be capable of being a vector. Many vectors of virus pathogens make little use of the crop, neither feeding on the plants nor using them for oviposition. Even though these insects will not be used in the subsequent transmission studies, there is a need to evaluate their ability to vector Ngs-phytoplasma. The ability of *Elasmocallus sp., Afrosus sp., Megalopa* and *Hecalus sp.,* and *Poophilus sp., to* vector Ngs-phytoplasma was not analyzed in this paper.

3.2 Development and evaluation of a loop mediated isothermal amplification of DNA (lamp)

3.2.1 Design and optimization of lamp primers

16S rDNA, Ngs-phytoplasma Kenya, sequence used in the development of LAMP primers is shown in Figure 9. The linear model of Ngs-phytoplasma 16S rDNA is shown in figure 10, while figure 11 is a model illustrating the process of Ngs-phytoplasma 16S rDNA stem-loop formation during LAMP cycling reaction . Four LAMP primers were developed in this study (Table 5). Primer Phyto-BIP consisted of the sequence (B1c) complimentary to B1 (24 nt), a TTT linker and a sense sequence of B2 complimentary to B2c (18 nt). Primer Phyto-FIP consisted of complimentary sequence of F1 (24nt), a TTT linker and the sense sequence of F2 (15 nt). Primers B3 and F3 consisting of the complimentary sequences of B3 and F3 regions of the targeted DNA respectively.

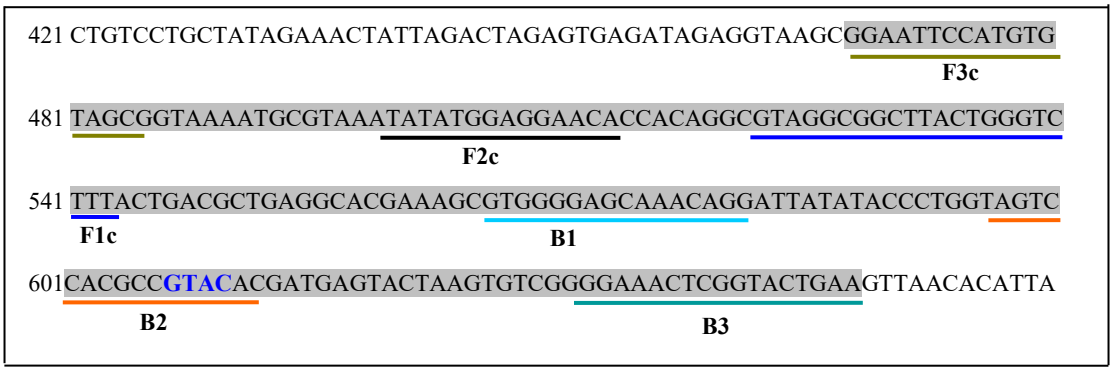


Fig. 9 Location and name of the regions targeted by LAMP primers in the sequence of Ngs-phytoplasma-Kenya (GenBank accession No. AY377874). The model was generated using PrimerAnneal bioinformatics tool (JustBio). Lines indicate the position on the target sequence where that LAMP primers anneal. The Bold-blue nucleotides indicate the *Rsa*I recognition site. Highlighted nucleotides represent the portion of the gene amplified by LAMP.

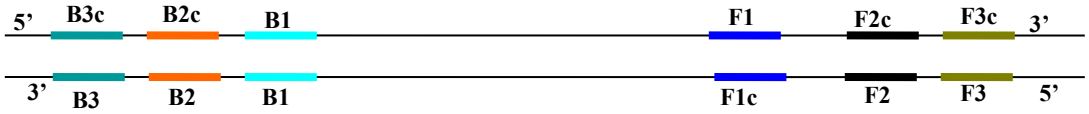


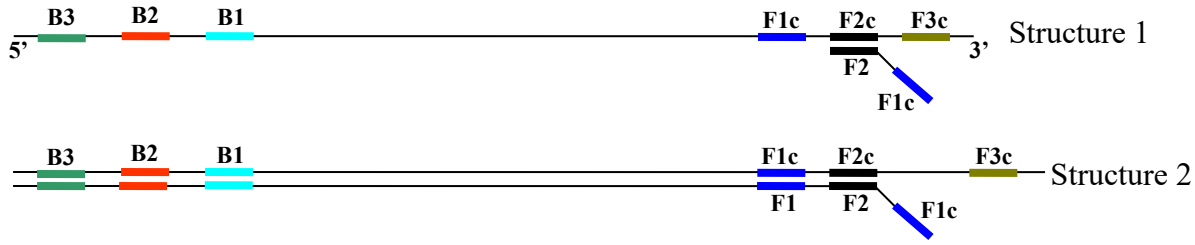
Fig. 10 Double stranded linear Model of NGS-phytoplasma 16S rDNA gene showing the regions recognized by FIP, BIP, F3 and B3 primers (Modeled by the Author)

Table 5. Oligonucleotide primers designed for Loop mediated isothermal amplification of DNA (LAMP)

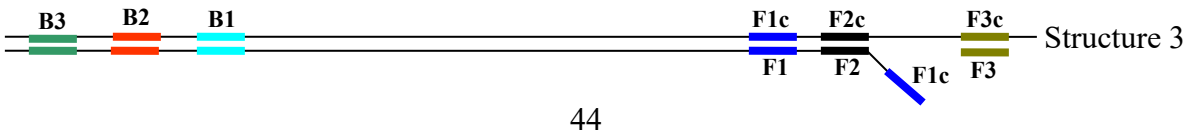
Primer Sequence	T_m (^{0}C)
Phyto-BIP 5'-CGTGGGGGCAAACAGGA-TTT-AGTACTCATCG TTTACGGC-3'	73.4
Phyto-FIP 5'-TCAGCGTCAGTAAAGACCCAG CGTTTATATGGAGGAACA CC-3'	73.3
Phyto-B3 5'- ACTTCAGTACCGAGTTTCC-3'	54.5
Phyto-F3 5'-CGGAATTCCATGTGTAGCG-3'	56.7

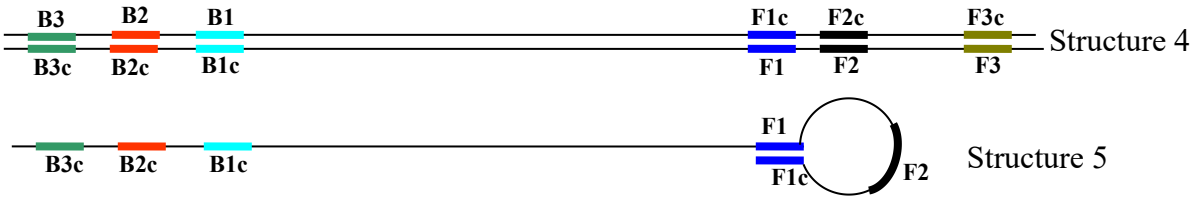
Fig. 11 Model of Ngs-phytoplasma Stem-Loop rDNA formation

a) The target DNA is denatured to a single strand by heating at 95^{0}C for 5 minutes (Structure 1). Phyto-FIP hybridizes to F2c region on the target DNA (structure 1) and initiates complimentary DNA strand synthesis forming structure 2.

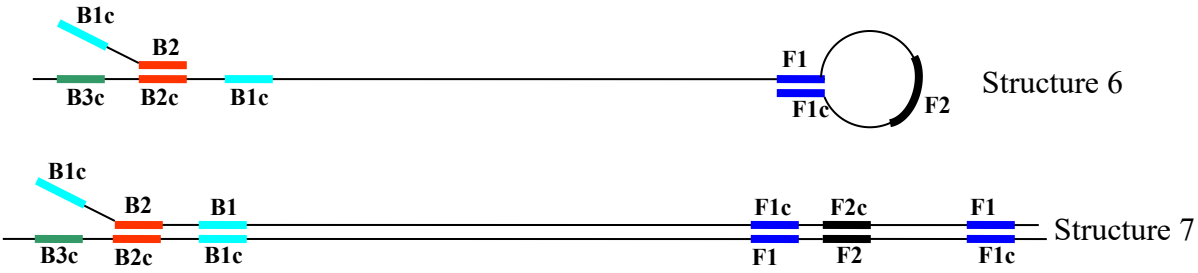


b) Phyto-F3 hybridizes to F3c in the target DNA (structure 3) and initiates strand displacement DNA synthesis, releasing a FIP-linked complimentary strand (which can form a looped outer structure at one end) (structure 5) and a double stranded DNA (structure 4)

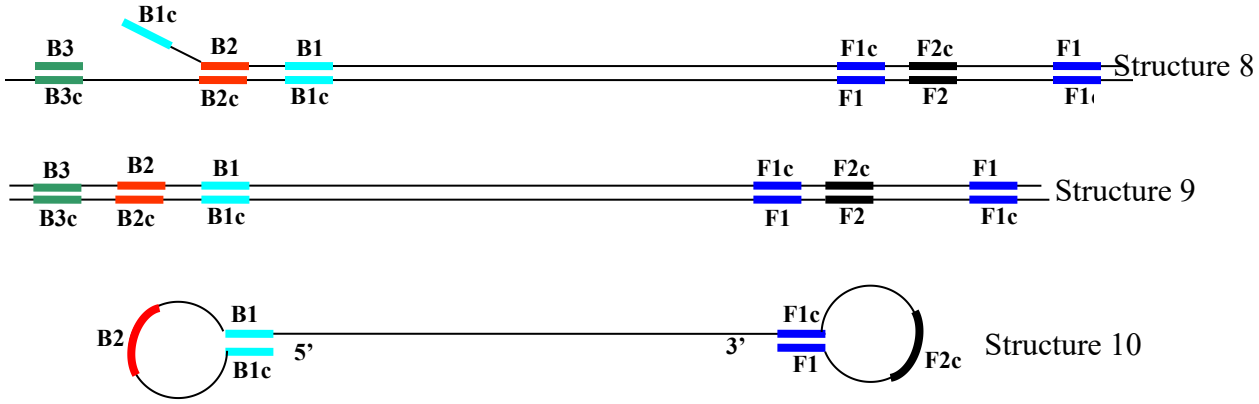




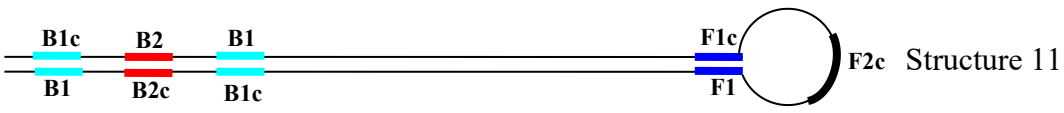
c) This single stranded DNA (Structure 5) serves as template for BIP-initiated DNA synthesis (structure 6) forming structure 7.



d) Phyto-B3 hybridizes to B3c region of structure 7 (structure 8) and initiates strand displacement DNA synthesis, leading to production of a dumb-bell form DNA (structure 10) and a double stranded DNA (structure 9)



e) The dumb-bell form DNA (structure 10) is quickly converted to a stem-loop DNA (structure 11) by self-primed DNA synthesis. This stem-loop DNA then serves as the starting material for LAMP cycling, the second stage of the LAMP reaction.



The stem-loop DNA (Structure 11) lack the B3 and F3 regions of the target DNA and their corresponding primers are redundant after the formation of stem-loop DNA (Structure 11, no B3 and F3 regions for the outer primers). This observation show that B3 and F3 primers are only involved in strand displacement DNA synthesis (Structure 3 and structure 8) in the initial stages of LAMP. After the formation of the stem-loop DNA (structure 11), the function of B3 and F3 primers cease. This account for why Phyto-B3 and Phyto-F3 primers were used in low concentrations (5 pmol of each) (refer 2.3.3 above). Structure 10 is gap repaired to form structure 11 (stem-loop DNA) by self-primed DNA synthesis from 3' to 5' ends. This self-priming DNA synthesis is a unique feature of LAMP.

Hybridization of the four primers to the target DNA in the initial step is critical for the stem loop formation in LAMP (Fig 12). Therefore, the primers should be chosen such that their melting temperatures (T_m) fall within certain ranges (Notomi *et al.,* 2000). The T_m values (Table 7) of the outer primers (Phyto-B3 and Phyto-F3) were set lower than those of inner primers (Phyto-FIP and Phyto-BIP) to ensure that synthesis occurred earlier from inner primers than from outer primers. In addition, the outer primers were used at 5:40 the concentration of the inner primers. Loop formation in LAMP is significant because the loop exposes the region of the target DNA where the primers anneal. Strand displacement DNA synthesis is a rate limiting step for amplification in LAMP, therefore, the size of the target DNA should be set to less than 300 bp (Notomi *et al.,* 2000). In this study, the LAMP primers were chosen such that about 182 bp (Fig. 9) of the Napier grass stunt phytoplasma rDNA would be amplified.

3.2.2 Examining specificity of lamp

When DNA extracted from Ngs-diseased plants were subjected to LAMP assay, Ladder-like DNA fragments ranging from about 182 bp to 400 bp confirmed the specific amplification of Ngs-phytoplasma rDNA (Fig. 12). No amplification was observed in the healthy Napier samples used as control, confirming the specificity of the amplification.

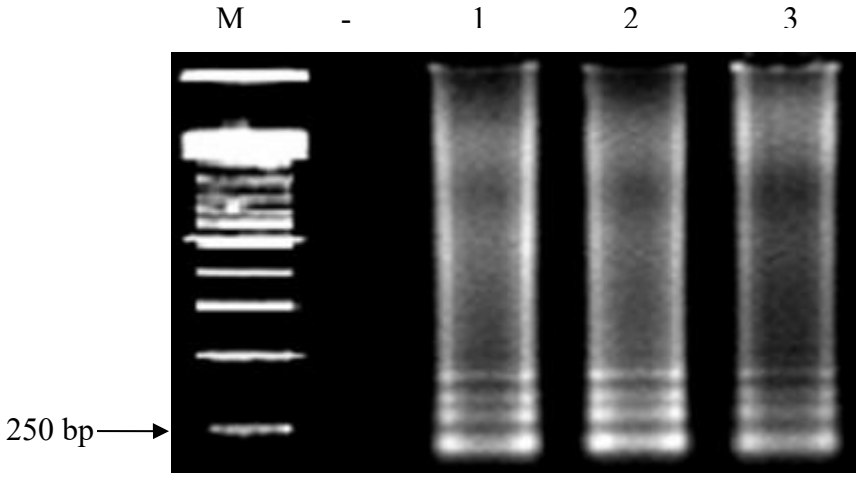


Fig. 12 Electrophoresis analysis of LAMP amplified products of Ngs-phytoplasma rDNA. Lanes; M – 1 kb ladder (Fermentas), (1-3), (+) = DNA isolated from diseased grasses, (-) = Negative control (DNA isolated from healthy Napier grass)

Apart from the successful negatively controlled LAMP (Fig. 12), the smallest structure expected in LAMP was about 182 bp, and was used to further strengthen specific amplification of Ngs-phytolasma rDNA. According to Fig. 12 above, the smallest band size produced was slightly below the 250 bp band of the 1 kb marker used to ladder this assay, and was estimated to be about 182 bp.

3.2.3 Examining sensitivity of lamp

Figure 13 and table 6 summarizes the results when LAMP technique was used to detect Ngs-phytoplasma rDNA in 4 samples of 10-fold serial dilutions. The results produced by 1st round and Nested PCR assays on the same samples (serial dilutions) are also included.

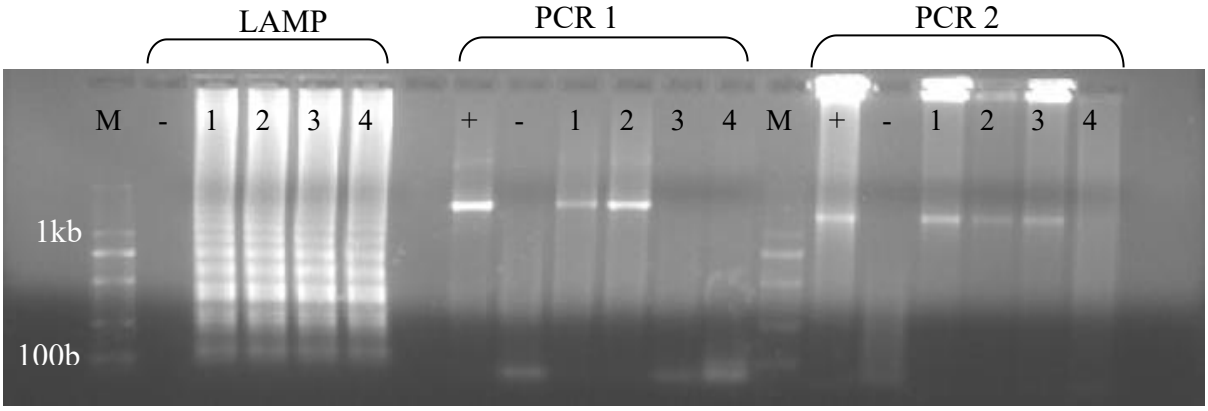


Fig. 13 Gel electrophoresis analysis of LAMP, One-round and Nested PCR for the detection of Ngs-phytoplasma rDNA in the 4 x 10^{-1}serial dilutions of Ngs-phytoplasma-Kenya. Lanes; M – 100 bp Mid Range ladder (Gelpilot), (-) = negative control (healthy sample), (+) = positive control, (1-4) = Serial dilutions 1-10^{-4}.

One round PCR amplified the target gene up to dilution 10^{-1}; Nested PCR increased the sensitivity to dilution 10^{-2} while LAMP assay further increased the detection limit to dilution 10^{-3} (Fi. 13, Table 6). Dilution 10^{-3} was negative by PCR (false negativity) yet it carried phytoplasma as detected by LAMP. Ngs-phytoplasma rDNA was present in solution, but the titre fell below the detection range of PCR. This observation score the analytical sensitivity anticipated in using LAMP as a detection tool, and serve to strengthen the fact that; PCR can give a false negative result if the amount of the targeted DNA template is low.

Table 6. Analytical sensitivities of One-round PCR, Nested PCR and LAMP for the detection of Ngs-phytoplasma rDNA in the serial dilutions.

Assay	NGS-phytoplasma 16S rRNA serial dilutions				
	10^{-3}	10^{-2}	10^{-1}	1	0
One round PCR	-	-	+	+	-
Nested PCR	-	+	+	+	-
LAMP	+	+	+	+	-

Using PCR analysis, 3 of 4 (75%) of the Napier grass DNA template dilutions were positive, whereas 4 of 4 (100%) were positive by LAMP (Table 6). Three Napier grass samples (75%) were positive by both Nested PCR analysis and LAMP; one sample (25%) was positive by LAMP and negative by PCR analysis; no sample (0%) was positive by PCR analysis and was negative by LAMP. No amplification was observed in the samples with zero targeted DNA (negative control) in all the three assays. The relative agreement between Nested PCR and LAMP was 80.0% (agreed in 4 out of 5 tests) (Table 6). This agreement between PCR analysis and LAMP was considered very good (80% agreement). The only discordant sample was dilution 10^{-3}, which was positive by LAMP but negative by PCR analysis. On the basis of the higher analytical sensitivity of the LAMP, it is likely that the discordant sample fell below the detection limit/threshold of PCR.

3.2.4 Molecular typing of lamp products

Digesting the LAMP amplicons with *Rsa1* restriction endonuclease produced exactly two bands (Fig. 14).

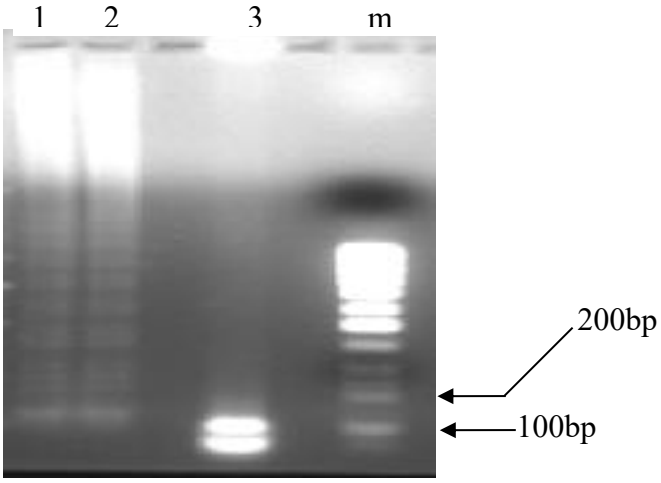


Fig. 14 Agarose gel electrophoresis result of LAMP amplicons digested with restriction enzyme *Rsa*1. Lanes: M- 100 bp marker (Gelpilot), 1 & 2- LAMP products before digestion, 3- LAMP product samples after Rsa1 digestion.

*Rsa*1 cuts B2 (Fig. 14). Consequently, if the LAMP products had the same structure as displayed in Fig. 14, the products would be fragmented by *Rsa*1 digestion into 141, and 41 bp. According to figure 14, the two bands produced by *Rsa*1 digestion were estimated to be about 141bp and 41 bp, quite in agreement with the predicted sizes. We can conclude that the LAMP amplified products, were rDNA from Ngs-phytoplasma.

LAMP was simple, did not require thermal cyclers, and the time required to arrange a LAMP reaction was very short as compared to PCR assay. I could only perform the reaction in a simple water bath, making LAMP ideal in resource-limited situations. An attractive feature of LAMP was its ability to generate large amounts of white magnesium pyrophosphate precipitate in positive reactions (data not shown), this enabled easy visual identification of positive reactions before gel electrophoresis. Being able to yield detectable copies of targeted DNA in only 60 minutes, The DNA amplification step was highly robust. Nevertheless, the assay had 10-fold higher analytical sensitivity than did the Nested PCR analysis (1 x 10-2 versus 1 x 10-3). The general agreement between LAMP and nested PCR was found to be excellent (80%). These features make LAMP a strong option for molecular diagnosis of NGS-Phytoplasma.

3.3 Acquisition and multiplication of Ngs-phytoplasma in the Napier canopy insects

3.3.1 Detection of phytoplasma in the insects immediately after acquisition access period

3.3.1.1 First round PCR

Five from ten test insects produced bands of about 1.5 kb by first round PCR in the pooled DNAs isolates, immediately after 20 days Acquisition Access Period (AAP) (Fig 15). The 5 insects which were positive by first round PCR were *Recilia banda, Exitianus 5, Cicadulina sp., Cofana spectra* and *Thriambus levus*. Phytoplasma titer in these insects immediately after the AAP was enough to be detected by one round PCR. It can be hypothesized that the 5 insects incriminated in this assay predominantly probed phloem, and thereby acquired a higher titer of phytoplasma in 20 days, detectable by first round PCR. The 20 days AAP was considered optimal for plant hoppers, which feed on both phloem and xylem, and therefore require prolonged feeding period to reach the phytoplasma acquisition level of leafhoppers, which are exclusively feeding on phloem.

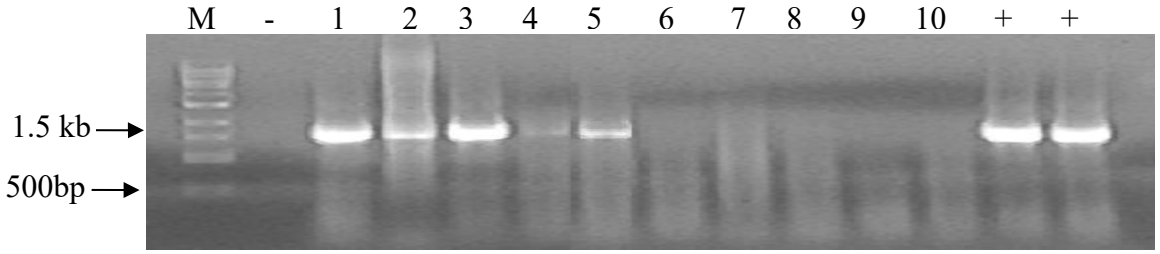


Fig. 15 Electophoresis of First round PCR products of insect samples screened for phytoplasma immediately after AAP. Lanes: M- 1 kb gene ruler (New England Biolabs), 1- *Recilia banda*, 2- *Exitianus sp 5,* 3- *Cicadulina sp., 4-Cofana spectra.,* 5- *Thriambus levus,* 6- *Rhinottetix sp*., 7- *Leptodelphax dymas*, 8- *Thriambus strenuous*, 9- *Sogatella manentho*, 10- *Glossocratus afzalii*, (-) = Negative control, (+)= positive control.

3.3.1.2 Nested PCR

This assay on first round PCR amplicons, after AAP increased the number of phytoplasma carrying insects from 5 to 7 out of the 10 tested (Fig 16). The two additional insects incriminated by this assay were *Rhinottetix sp,* and *Glossocratus afzalii*. The phytoplasma titre in the two insects fell below the detection threshold of first round PCR but were within the detection range of Nested PCR. This observation score the strength of Nested PCR to increase the sensitivity of phytoplasma detection as originally anticipated by Deng and Hiruki, (1999). All the five insects whose phytoplasma titre were detected in the first round PCR, were all positive in Nested PCR. Meaning that the amplifications were indeed from phytoplasma rDNA.

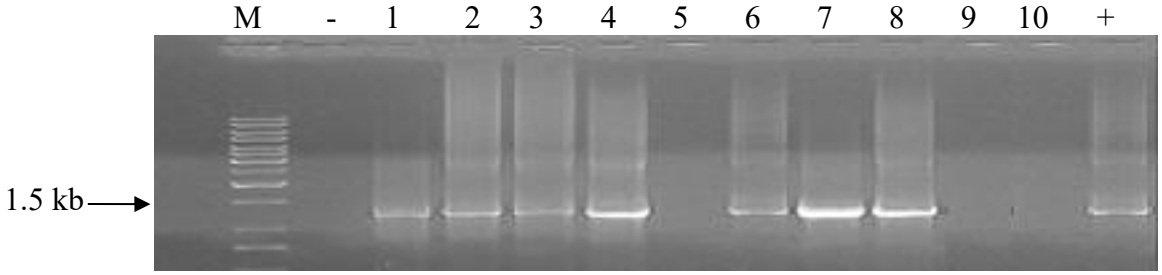


Fig. 16 Electrophoresis of Nested PCR roducts of test insects after AAP. Lanes: M- 500 bp ladder (Gelpilot), 1- *Recilia banda*, 2- *Exitianus sp 5*, 3- *Cicadulina sp*., 4-*Cofana spectra*., 5- *Thriambus strenous*, 6- *Rhinottetix sp.,* 7- *Glossocratus afzalii*, 8- *Thriambus levus*, 9- *Sogatella manentho*, 10- *Leptodelphax dymas*, (-) = Negative control, (+)= positive control.

The titer of Ngs-phytoplasma in *Leptodelphax dymas, Sogatella manentho*, and *Thriambus strenuous* were undetectable by both first round and Nested PCR (Fig. 15 and 16). These insects, being plant hoppers probe both phloem and xylem, therefore only switching to phloem occasionally compared to leafhoppers whose diet is majorly phloem photo assimilates. After 20 days of rearing on diseased Napier, their phytoplasma titers were still very low. They

required longer AAP days to acquire phytoplasma levels detectable by PCR. However, negativity by nested PCR could not mean that the insects carried no phytoplasma, but that the phytoplasma level fell below the detection range of PCR.

3.3.1.3 Loop Mediated Isothermal Amplification of DNA (LAMP).

Pooled DNA isolates from all the insects used in this experiment tested positive with LAMP immediately after 20 days AAP (Fig. 17). This observation scored the point that LAMP is a very sensitive phytoplasma diagnostic tool, detecting phytoplasma at lower levels undetectable by PCR. Through LAMP technique, we found that *Leptodelphax dymas, Sogatella manentho,* and *Thriambus strenuous* indeed carried phytoplasma, rescuing these insects from rejection, of not acquiring phytoplasma during feeding, and therefore not being able to vector Ngs-phytoplasma.

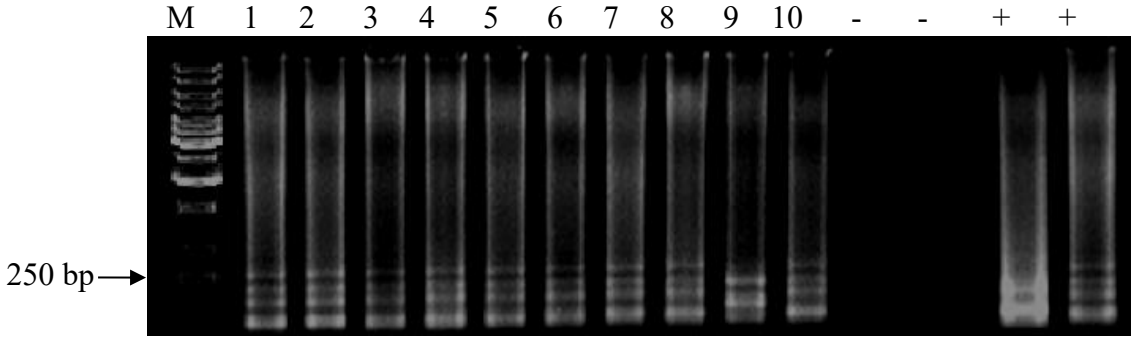


Fig. 17 LAMP products of insect samples immediately after AAP analysed by agarose gel electrophoresis. Lanes: M- 1 kb ladder (New England Biolabs), 1- *Recilia banda*, 2- *Exitianus 5*, 3- *Cicadulina sp*., 4- *Cofana spectra*., 5- *Thriambus light*, 6- *Rhinottetix sp.,* 7- *Leptodelphax dymas,* 8- *Thriambus strenuous*, 9- *Sogatella manentho*, 10- *Glossocratus afzalii*, (-) = Negative control, (+)= positive control.

The result on phytoplasma titre, in the test insects immediately after AAP suggest that all the insects tested probe phloem at some point during their feeding (Fig. 17). However, *Recilia banda, Exitianus 5, Cicadulina sp., Cofana spectra* and *Thriambus levus,* tend to feed more on phloem than all the other insects, they were able to pick larger titres of phytoplasma detectable by first round PCR (Fig. 15), after only 20 days AAP. If quantity of phytoplasma ingested during feeding is significant for transmission, we can suspect a vector species among the four insect species. However, as confirmed by LAMP, all the ten insects used in this experiment carried Ngs-phytoplasma after 20 days AAP.

Phloem-feeding insects acquire phytoplasmas passively during feeding in the phloem of infected plants. The feeding duration necessary to acquire a sufficient titer of phytoplasma is the acquisition access period (AAP). Acquisition of phytoplasma is significant for transmission. After acquisition, a vector species should support the multiplication of pathogen, and eventually transmit it during feeding. This assay therefore did not incriminate and insect as a vector of Ngs-phytoplasma, but provided an insight into the feeding behaviour of insects sampled from Napier canopy.

3.3. 2 Detection of phytoplasma in the insects after 30 days rearing on healthy plants.

Relocating the test insects from diseased to healthy grasses was done to; a) stop the insects from ingesting any more phytoplasmas, b) clean the gut of any previously ingested phytoplasma and c) allow the already ingested pathogens to interact in a circulative manner with the vector of NGS-phytoplasma.

3.3.2. 1 First round PCR

No phytoplasma was detected in the pooled DNAs of the 10 test insects by first round PCR (Fig. 18), when the test insects were reared on healthy grasses for 30 days. Looking at Figure 18, two positive controls were used, one positive control did not produce any band, and the band in the second control was really faint, meaning that some factor might have affected the efficiency of first round PCR, a common problem in many PCR assays. However, the phytoplasma titer might have regressed to below the detection threshold of first round PCR.

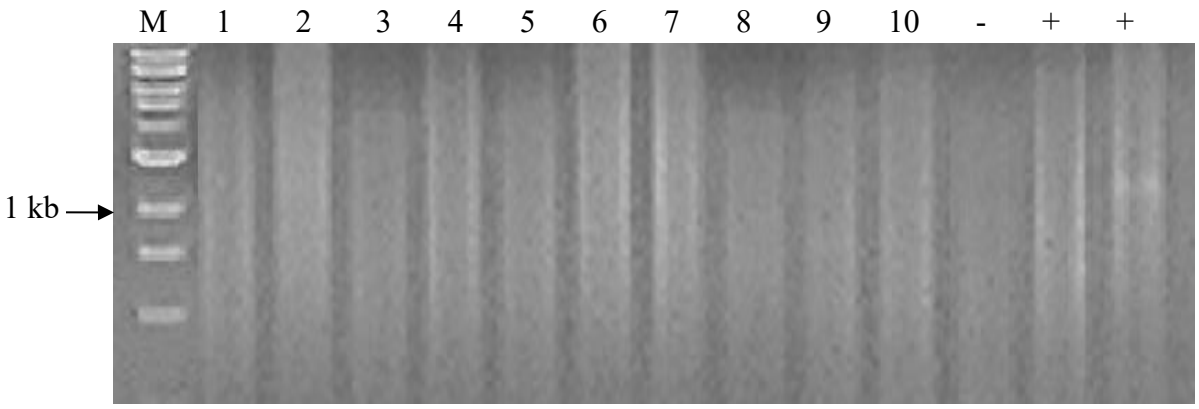


Fig. 18 Electrophoresis result of first round PCR products of DNA isolated from the test insects after 30 days latency period. Lanes: M- 1 kb gene ruler (New England Biolabs), 1- *Recilia banda*, 2- *Exitianus 5*, 3- *Cicadulina sp*., 4-*Cofana spectra*., 5- *Thriambus levus*, 6- *Rhinottetix sp*., 7- *Leptodelphax dymas*, 8- *Thriambus strenuous*, 9- *Sogatella manentho*, 10- *Glossocratus afzalii*, (-)= negative control, (+)= positive control

3.3.2.2 Nested PCR

Amplification of a phytoplasma-characteristic 1.2-kb 16 S rDNA fragment in the nested reactions primed by R16F2n/R16R2 confirmed that *Cofana spectra, Cicadulina sp., Exitianus 5, Rhinotetix and Thriambus levus* were infected by a phytoplasma after 30 days of rearing on healthy Napier grass (Fig. 19). Habouring phytoplasmas, these insects were able to support multiplication of Ngs-phytoplasma and therefore can vector the pathogen. The other 5 insects tested were all negative. If vectors, we expected the phytoplasma titre in the insects to rise at least to the detection level of Nested PCR. *Recilia banda, Sogatella manentho, Leptodelphax dymas, Glossocratus afzalii* and *Thriambus strenuous* were declared unable to vector NGS-phytoplasma.

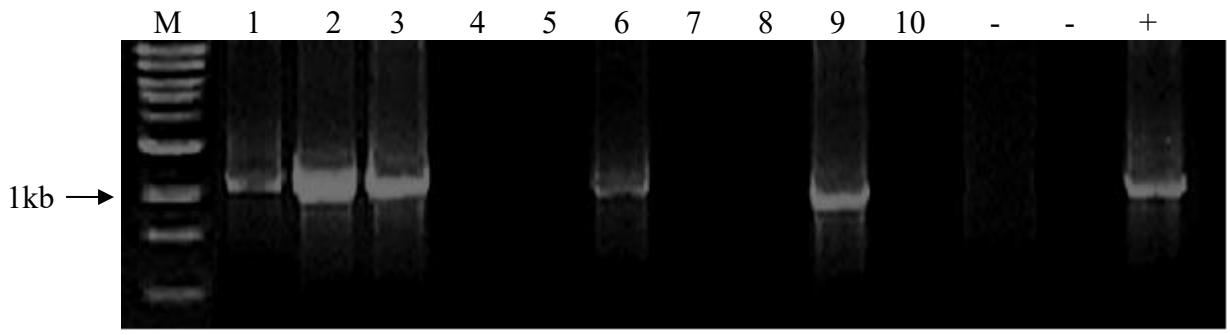


Fig. 19 Result of DNA isolated from the insects immediately after 30 days AAP, amplified by Nested PCR and analyzed by agarose gel electrophoresis. Lanes: M- 1kb gene marker (New England Biolabs), 1- *Cofana spectra,* 2- *Cicadulina sp.,* 3- *Rhinotettix sp*., 4- *Recilia banda*, 5- *Leptodelphax dymas,* 6- *Thriambus levus*, 7- *Sogatells manentho ,* 8- *Thriambus strenuous*, 9- *Exitianus sp 5,* 10- *Glossocratus afzalii.*

3.3.2.3 Lamp

LAMP technique on the pooled DNA extracted from test insects after 30 days on healthy plants, revealed ladder like DNA fragments ranging from about 182 to 400 bp confirming that *Cofana spectra, Cicadulina sp., Exitianus 5, Rhinotetix, Thriambus levus* and *Thriambus strenuous* were infected with phytoplasma (Fig. 20). This observation raised the number of phytoplasma carrying insects to 6 out of 10. The other four insects; *Leptodelphax dymas, Sogatella manentho, Glosocratus afzalii* and *Recilia banda* tested negative, carrying phytoplasmas (if any) below the detection limit of LAMP, on the sense of higher analytical sensitivity of LAMP, these insects were regarded phytoplasma negative, and were exonerated as possible vectors of Ngs-phytoplasma,

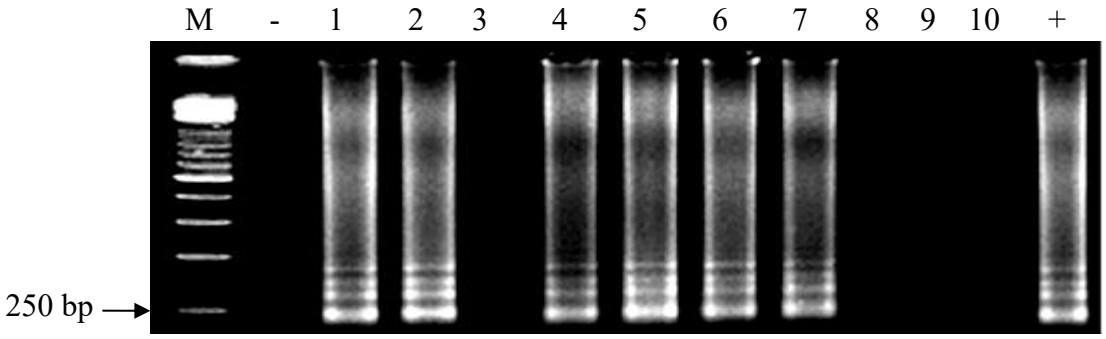


Fig. 20 LAMP products analyzed by Agarose gel electrophoresis. Lanes: M- 1 kb ladder (Fementas), 1- *Exitianus sp 5*, 2- *Rhinotettix sp*., 3- *Sogatella manentho*, 4- *Cicadulina sp.,* 5- *Cofana spectra*, 6- *Thriambus levus*, 7- *Thriambus strenous.,* 8- *Leptodelphax dymas,* 9- *Recilia banda*, 10- *Glossocratus afzalii*, (-)= negative control, (+)= positive control

The table 7, below summarizes the results obtained when the individual insects from each pool (n = 5) was screened for the presence of phytoplasma by LAMP, after 30 days of rearing on healthy Napier. The results show that 4 individuals out of 5 (80%) of *Cofana spectra* (Fig.

21) screened using LAMP assay carried phytoplasma (Table 7). This study incriminated *Cofana spectra* as the most probable vector of Ngs-phytoplasma. Other probable vectors which cannot be ruled out are *Cicadulina sp.,* (60%), *Exitianus sp. 5* (40%), *Thriambus levus* (40%), *Thriambus strenuous* (20%) and *Rhinottetix sp.,* (20%).

Fig. 21 *Cofana spectra*, the white leafhopper (Picture courtesy of Jimmy Pitchar, ICIPE*,* 2008)

Table 7. Summary of individual insects screened for phytoplasma, using LAMP technique, after 30 days of rearing on healthy grasses.

Insects tested	# LAMP positive (n = 5)
Recilia banda	0
Exitianus sp5	2
Cicadulina sp	3
Cofana spectra	4
Thriambus levus	2
Thriambus strenuous	1
Rhinottetix sp.	1
Sogatella manentho	0
Leptodelphax dymas	0
Glossocratus afzalii	0

It can be hypothesized that the phytoplasma titer found in *Leptodelphax dymas, Sogatella manentho, Recilia banda, Glossocratus afzalii* after AAP represents the amount of pathogen ingested with the phloem sap (Weintraub and Beanland, 2006). A first multiplication may happen in the gut. Only in few individuals the pathogen reaches the specific regions inside the insect where the main multiplication takes place and, thus, the titer increases. In the present experiment (Table 7), this was only observed for *Cofana spectra* (4 out of 5 insects), *Cicadulina sp.* (3 out of 5 insects)*, Thriambus levus* (2 out of 5 insects) and *Exitianus sp. 5* (2 out of 5 insects). These insects were seen to host the pathogen 30 days post acquisition period and therefore could transmit Ngs-phytoplasma. LAMP positive products witnessed with *Thriambus strenuous* (1 out of 5 insects) and *Rhinottetix sp* (1 out of 5 insects), was probably due to contamination, a big problem of this assay. Because of the high analytical sensitivity, a slight cross-contamination of the samples could lead to a positive LAMP reaction.

An insect unable to sustain multiplication of a phytoplasma will not serve as a vector. On the other hand, multiplication of phytoplasmas in insects does not always result in infective vectors (Purcell *et al*., 1981). This study conclude that *Leptodelphax dymas, Sogatella manentho*, *Recilia banda* and *Glossocratus afzalii* are not vector(s) of Ngs-phytoplasma because they could not sustain multiplication of the pathogen.

While working with Chrysanthemum Yellow (CY) phytoplasma, vectored by 3 insect species, Domenico *et al., (2007) showed that the multiplication pattern of the same phytoplasma can be different in insect vector species. Macrosteles*

quadripunctulatus supported the highest number of Chrysanthemum Yellows phytoplasma cells per DNA unit and multiplication was much faster than in *Euscelidius incisus* and *Euscelidius variegatus*. The rate of multiplication is correlated with the incubation time in the insect; actually, latency in *Macrosteles quadripunctulatus* lasts 18 days, while in *Euscelidius variegatus* 30 days are required under the same conditions (Bosco *et al.*, 1997). A faster multiplication in the insect should result in an earlier invasion of the salivary glands and therefore, in a shorter latent period. Achieving infection rate of 80% (Table 7), multiplication of Ngs-phytoplasma was considered very fast in *Cofana spectra* than all the insects tested. This study incriminated *Cofana spectra* as the most probable vector of Ngs-phytoplasma.

3.4 Membrane feeding assay

3.4.1 Nested PCR on the pooled DNA isolate from the feeding medium

Nested PCR using the phytoplasma universal primer pair P1/P6 and R16F2n/R16R2 amplified about a 1.2 kb DNA band confirming the presence of phytoplasma in 4 out of 12 pools of sucrose-TE rations fed to the test insects (Fig. 22). Insects whose corresponding pooled DNAs were PCR positive included *Cofana spectra, Exitianus sp. 5, Cicadulina sp.,* and *Thriambus levus*.

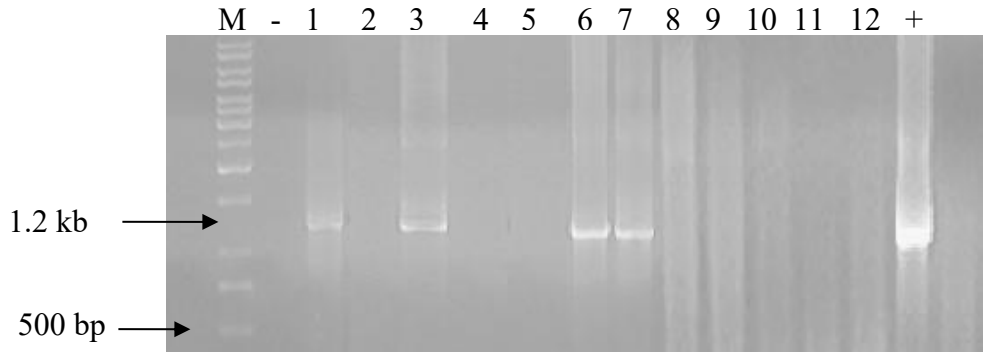


Fig. 22 Electrophoresis result of Nested PCR products of DNA isolated from sucrose-TE. M- 1kb gene ruler (New England Biolabs), (-)= negative control, 1- *Cofana spectra*, 2- *Cofana unimaculata*, 3- *Exitianus 5*, 4- *Cofana polaris*, 5- *Recilia banda*, 6- *Cicadulina sp.*, 7- *Thriambus levus*, 8- *Thriambus strenuous*, 9- *Rhinottetix sp.,* 10- *Glossocratus asfazelli*, 11- *Leptodelphax dymas,* 12- *Sogatella manentho.*

3.4.2 Determining the transmission rate of Ngs-phytoplasma

Table 8 summarizes the results produced when the individual insects from the Nested PCR positive pools of DNA isolated from sucrose-TE were analyzed for phytoplasma presence by Nested PCR (Gel photos not shown).

Table 8. Number of Sucrose-TE rations exposed to each insect and the number positive when individual DNAs from the positive pools were subjected to nested PCR. Only Sucrose-TE from the positive pools are shown.

Species	*# Membrane feeding assays*	*# Membrane feeding assays positive*	*Transmission rate*
Cofana spectra	6	2	32%
Cicadulina	6	1	16%
Exitianus sp. 5	6	1	16%
Thriambus levus	6	0	0

Sucrose-TE ration fed on by *Thriambus levus* was phytoplasma positive in the pooled DNA (Fig 24 above), but was negative when the individual insects were screened by PCR. The PCR positive result could be due to cross contamination between the samples. After several PCR trials (up to nested 2), only 1 insect from *Cicadulina sp.,* and *Exitianus sp. 5* (Fig. 23) pools were phytoplasma positive, transmitting NGS-phytoplasma to about 16% of the sucrose-TE rations. Their chances to vector Ngs-pytoplasma was considered very narrow.

Fig. 23 Adult *Exitianus sp. 5* (Picture courtesy of Jimmy Pitchar, ICIPE, 2008)

Transmitting Ngs-phytoplasma to 32% of the sucrose-TE rations (Table 8), *Cofana spectra* was considered inoculative. In the same manner, it can transmit the pathogen. The chances of *cofana spectra* transmitting Ngs-phytoplasma to susceptible grasses was considered very high, and this study incriminated *Cofana spectra* as the vector of Ngs-phytoplasma. In the next topic, natural transmission experiment shall be carried out to confirm the vector identity of *Cofana spectra.*

3.4.3 Molecular typing of the nested PCR products

Nested PCR amplicons of the DNA isolated from the sucrose-TE ration fed on by *Cofana spectra* was used to characterize phytoplasmas obtained from the sucrose-TE feeding medium. Figure 24 shows the results when *Alu*1 and *Rsa*1 digests were separated in a 1.5% Agarose gel stained with Ethidium bromide.

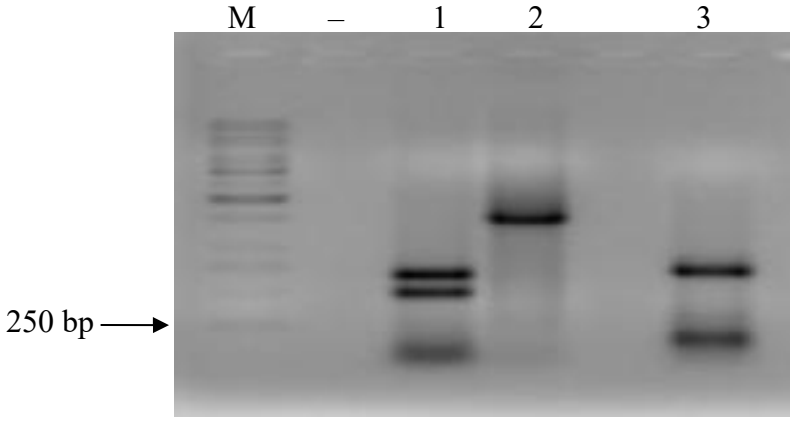


Fig. 24 Electrophoresis analysis of Nested PCR products digested with *Alu*1 and *Rsa1* restriction enzymes. Lanes: M1- 1kb gene ladder (New England Biolabs), (-) = negative control (water), 1- *Rsa*1 digested PCR product, 2- undigested PCR product, 3- *Alu*1 digested PCR product.

According to the hypothetical restriction map (Fig. 25) developed by Arocha *et al.* (2006, Unpublished), *Rsa*1 cuts tRNA region of NGS-phytoplasma, Kenya rDNA (1400 bp) into; 700, 600 and four other fragments below the range of 1 kb gene ruler (Fig. 25). In the virtual digestion (Fig. 24), *Rsa*I produced 3 fragments of approximately 700, 600 base pairs. The other band produced was way below the range of a 1kb ladder, quite in agreement with the predicted sizes.

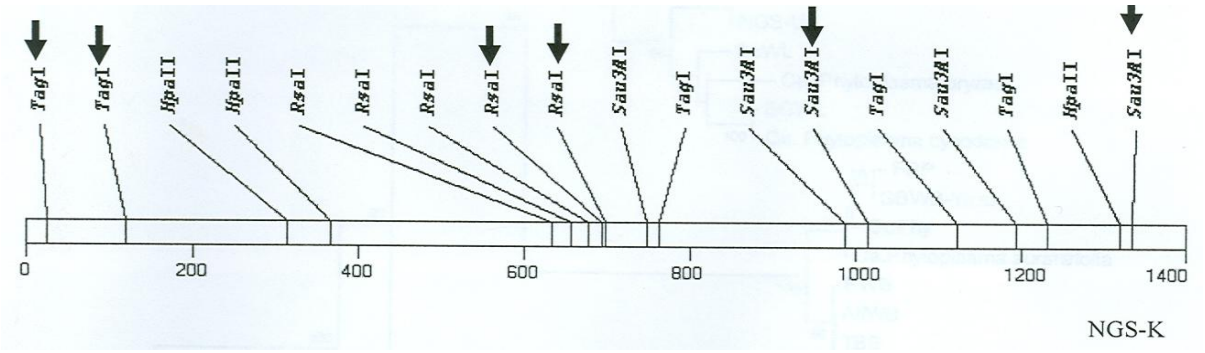


Fig. 25 Putative restriction site map of Ngs-phytoasma Kenya 16S rDNA (Arocha *et al.,* 2006, Unpublished)

Nei and Li similarity coefficient (F) between the *Rsa*1 RFLP patterns in the Arocha *et al.,* (2007) map (Fig. 25) and the actual digestion of Ngs-phytoplasma-Kenya 16S rDNA sequence is summarized in the table 9.

Table 9. Summary of Nei and Li similarity coefficient between the rDNA isolated from sucrose-TE ration (y) and the *Rsa*1 RFLP map in the Arocha *et al.* (2007) (Unpublished) (x).

	Nx	Ny	Nxy	F= 2Nxy/Nx+Ny
x	5		3	0.75
y		3		

Where; x - RFLP patterns in the Arocha *et al.* (2006) map

y - Actual digestion of 16S rDNA of NGSP-Kenya

Nx – Number of fragments in the the Arocha *et al.,* (2006, unpublished) map

Ny – Number of fragments obtained in the actual digestion of NGSP-Kenya rDNA.

Nxy – Number of fragments shared by the two strains

Based on the similarity coefficient (F) of 0.75 (high similarity coefficient) (Table 9), the study conclude that, the 16S rDNA isolated from the sucrose-TE ration of *Cofana spectra* was 75% similar to that of Ngs-phytoplasma 16S rDNA. The distance (D) between the two strains, 1-F, was found to be 0.25 or 25%, showing that the two strains were very close. This study concluded that the phytoplasma rDNA isolated from the sucrose-TE ration fed to by *Cofana spectra* was from Ngs-phytoplasma-Kenya.

3.5 Natural transmission experiment

LAMP technique was adopted for its sensitivity because phytoplasma rDNA in the test plants were hypothesized to be below the detection threshold of PCR since the plants were only 90 days post acquisition period. The sense of performing acquisition, latency and transmission in the same cage was that, the insect vector would pick phytoplasma from the diseased plant, the pathogen would replicate in a vector species which then becomes inoculative. Wei *et al.* (2004) determined that there was a six fold increase in onion yellows phytoplasma per week in the plant after inoculation, therefore, the 90 days incubation period was enough to build enough titre of phytoplasma detectable by LAMP.

3.5.1 Lamp assay on pooled DNA of exposed plants

Cofana spectra and *Exitianus spe. 5* were pooled twice (Fig. 26) because they had many replications (Table 4). Ladder like DNA fragments ranging from about 182 to 500 bp on a 1% agarose gel (Fig. 26) were amplified from DNA extracted from a plants fed on by *Cofana spectra, Cicadulina sp. Extianus sp. 5, Thriambus levus and Rhinottetix sp.*

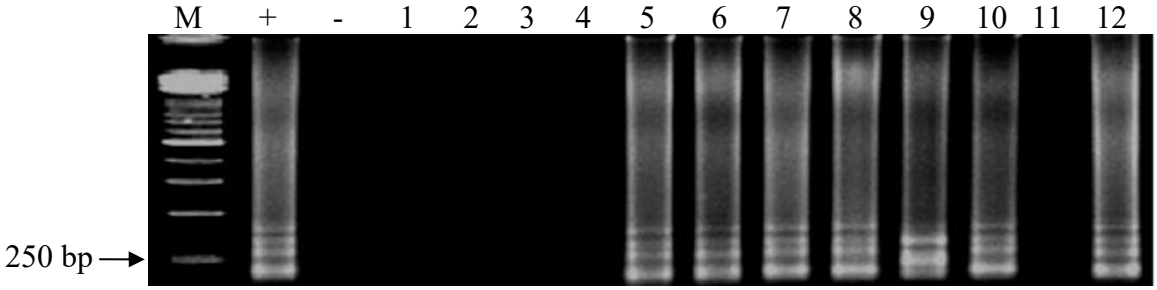


Fig. 26 Agarose gel (1%) electrophoresis of LAMP products on pooled DNAs; extracted from exposed plants. Lanes: M = 1 kb ladder (Fermentas), (-) = negative control, 1- *Leptodelphax dymas*, 2- *Sogatella manentho,* 3- *Glossocratus afzalii*, 4- *Thriambus strenuous*, 5- *Cofana spectra*, 6- *Cofana spectra*, 7- *Thriambus levus*, 8- *Cicadulina sp.*, 9- *Exitianus sp. 5*, 10- *Exitianus sp 5*, 11- *Recilia banda*, 12- *Rhinotettix sp.*

The presence of the expected sized band in electrophoresis (182 bp), estimated by the fragment of DNA that is isolated in the primer design process (1.2 above), was enough for identification of Ngs-phytoplasma infection in the diseased plants. No amplification was observed on the negative control and plants fed on by 5 other insect species used in this assay (Fig. 28).

3.5.2 Determining Ngs-phytoplasma transmission rate

Conducting LAMP assay on individual plants (Table 10) from the positive pools showed that *Cofana spectra* is the leading vector (27% transmission rate) of Ngs-phytoplasma . Other vectors include *Exitianus sp. 5* (10%) and *Cicadulina sp.* (24%)

Table 10. Results of Natural transmission experiment.

Insect	# plants exposed	# plants positive	Transmission rate (%)
Cofana spectra	61	17	27
Cicadulina sp.	37	9	24
Exitianus sp 5	30	3	10
Thriambus levus	36	0	0
Rhinottetix sp.	19	0	0

Plants exposed to *Rhinotettix sp.*, and *Thriambus levus* all tested negative (Table 10). The positive result in their pooled DNAs (Fig. 26) of these plants was predicted to be due to contamination.

Among phytoplasma insect vectors known, the superfamily containing the largest number of vector species is the Membracoidea, within which all known vectors to date are confined to the family Cicadellidae (leafhoppers), subfamily Deltocephalinae (Tanne *et al.,* 2001), where *Cofana spectra, Cicadulina* and *Exitianus sp. 5,* incriminated in this study to be vectors of Ngs-phytoplasma belong. The ability of most members of Cicadellidae to interact with phytoplasma shows a longer evolutionary relationship between the two organisms (Domenico *et al.,* 2008).

Through LAMP, E-plants (Exposed plants) examined in this study were infected, but none of them showed any symptoms of Ngs-disease. This shows that in the Ngs-disease epidemiology, symptom-less but infected grasses do really exist, unnoticed by farmers.

4. CONCLUSION AND RECOMMENDATION

The long-term goal of this work has been to understand the epidemiology of Ngs-disease, an increasingly destructive disease of Napier grass in Western Kenya. Of particular importance has been the identification of insect vector species, in order to develop disease management strategies for growers. Work to identify the insect species responsible for vectoring NGS-phytoplasma has included field surveys (Bungoma District) and transmission tests at ICIPE's TRO campus Mbita (Western Kenya).

This study has made significant progress in determining the suite of candidate vector species found in and around Napier grass. Napier canopy was found to support 21 Homopteran insect species. Little is known why the grass plays a host to such a great community of insects, making it vulnerable to insect transmitted pathogens such as phytoplasmas. A clever guess is the sugar-rich phloem sap. With intensification of Napier grass farming in Kenya, we expect new Napier susceptible diseases to emerge and spread. A database search conducted by Daniel Masiga (ICIPE Scientist) revealed that the 16SrXI strain causing Napier stunt disease in Western Kenya is very closely related to strains isolated from various grasses, including Bermuda grass (accession. No. EF44448_1), sorghum grassy shoot phytoplasma (acc. No. AF550985_1), sugarcane white leaf phytoplasma (AB052874_1), and various unclassified strains. The potential of phytoplasma to infect crops and grasses other than Napier grass poses a great risk to the well being of agricultural communities in the eastern Africa where sorghum and sugarcane are major food and cash crops.

To support transmission experiments, this study also established a LAMP (Loop Mediated Isothermal Amplification of DNA) based phytoplasma detection scheme. LAMP is a nucleic acid amplification technique originally developed by Notomi *et al.* (2000). This study found this technique to be simple, rapid, sensitive and specific diagnostic tool for detection of phytoplasmas. LAMP was in good agreement with PCR, and detected phytoplasmas below the detection threshold of PCR. This is the first report of a LAMP-based phytoplasma detection technique. Further improvements are still needed, but adoption of LAMP as an Ngs-phytoplasma detection technique should be adopted.

Membrane feeding biossay using sucrose-TE ration was done using 12 homopteran insects sampled from Napier canopy. Using this assay, *Cofana spectra* was found to be the leading vector of NGS-phytoplasma, inoculating up to 4 out of 5 sucrose-TE food rations sampled. Although used majorly in this study as a simple technique to test transmissibility of NGS-phytoplasma, this assay was found to be excellent in isolating whole phytoplasma genome from the salivary glands of infective leafhoppers, therefore shedding light into the development of monoclonal antibodies for use in the diagnosis of Ngs-phytoplasma, a prompt into ELISA based Ngs-phytoplasma detection technique.

Standard biological transmission experiments were conducted using 10 insects. This assay confirmed that the white leafhopper, *Cofana spectra*, is the vector of Ngs-phytoplasma though *Cicadulina sp.,* and *Exitianus sp. 5* cannot be ruled out as additional vectors. Incrimination of 3 species of insects as vectors of a disease is of great epidemiological importance. However, a lot more research should be done to determine the latency period of NGS-phytoplasma in

each insect vector and how long do the insects remain inoculative. Incrimination of three vector species in this study shows that the vector specificity of Napier Grass Stunt Phytoplasma is not very strict. Future incrimination of additional vectors are therefore likely. Due to limitation of time, I did not test inoculative abilities of 11 homopteran insects sampled from Napier grass (Table 3). It is my recommendation that these insects should be screened and their phytoplasma vector status known.

Findings from this study should prompt the next stage of research and development on the management of Ngs-disease, insect-pathogen interactions and other aspects of vector biology. The results of this work should be published in scientific journals as a major contribution to the understanding of the pathogen and vectors for Ngs-disease. Consideration should be given to allocating a modest level of funding to the present project team in order to study aspects of insect vector management and breeding for resistance.

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