Molecular Determination and Characterization of Phytoplasma 16S rRNA Gene in Selected Wild Grasses from Western Kenya

Adam OJ1, Midega CAO1, Runo S2 and Khan ZR1

1International Centre of Insect Physiology and Ecology (icipe), Nairobi, Kenya
2Kenyatta University, Nairobi, Kenya

Abstract

Napier grass (Pennisetum purpureum) production for zero grazing systems has been reduced to rates of up to 90% in many smallholder fields by the Napier stunt (Ns) disease caused by phytoplasma sub-group 16SrXI in western Kenya. It is hypothesized that several other wild grasses in Kenya could be infected by phytoplasmas that would otherwise pose a significant threat to Napier, other important feeds and food crops. This study therefore sought to detect and identify phytoplasma strains infecting wild grasses in western Kenya using 16S ribosomal RNA (ribonucleic acid) gene as well as identify wild grass species hosting phytoplasmas in 646 wild grass samples that were collected in October 2011 and January 2012 during a random crosssectional survey conducted in Bungoma and Busia counties of western Kenya. DNA was extracted and nested polymerase reaction (nPCR) used to detect phytoplasmas. Two sub-groups of phytoplasmas were detected in eight grass species observed to grow near infected Napier fields. Only one of the two phytoplasmas reported was related to the Ns phytoplasma. There was a strong association between proportions of phytoplasma infection and the grass species collected (p = 0.001). C. dactylon, D. scalarum, B. brizantha, poverty grass and P. maximum had high proportions of infection and were abundantly distributed in western Kenya hence considered wild phytoplasma hosts. E. indica and C. ciliaris were scarcely distributed and had low infection rates. There was statistically significant difference in proportions of infection per location of survey (p = 0.001). Phytoplasma subgroups 16SrXI and 16SrXIV were the only phytoplasma genotypes distributed among wild grasses in western Kenya. Phytoplasma subgroup 16SrXIV predominantly infects only C. dactylon and B. brizantha grasses while phytoplasma subgroup 16SrXI is broad spectrum and infects a large number of wild grasses. In general, there is a diversity of wild grasses hosting phytoplasmas in western Kenya. These host grasses may be the reason for the high rates observed in the spread of Ns disease in western Kenya by acting as reservoirs for Ns phytoplasma.

Keywords: Phytoplasma; Pennisetum purpureum; Characterization; Napier stunt disease; Cynodon dactylon, Brachiaria brizantha, Digitaria scalarum

Introduction

Napier grass (Pennisetum purpureum) is an indigenous tropical African clumping grass which grows up to 5 meters tall. It is mainly vegetatively propagated through cuttings of about 3 to 4 centimeters in length and clump splitting. It has been widely used as fodder crop and for environmental sustenance, by stabilizing soils as well as acting as windbreaks [1]. In Kenya, napier grass has been employed in a new ‘Push-Pull’ management strategy for maize stem borers [2].

Napier grass has been used by many farmers in Kenya as the major livestock feed. It is as well sold to generate additional revenue. The increased population results in land subdivision which decreases farm size, hence, resulting in the adoption of the zero grazing system by most farmers which uses large amounts of fodder such as napier and other wild grasses that are cut and carried home for stall feeding [3].

A disease that attacks and greatly reduces the productivity of napier grass has been identified particularly in regions of western Kenya. Napier stunt disease (Ns) is a newly identified disease caused by a phytoplasma that adversely affects napier production at a rate between 30% and 90% observed in many smallholder fields. The year 2004, the disease is estimated to have affected over 23,298 km² of napier grass crop, an estimated 2 million households (about 30% of the population) in Western and Rift Valley provinces of Kenya [3].

Many grass diseases across the world have been attributed to phytoplasma infection. Four varieties of phytoplasmas were identified in seven species of grasses growing near sugarcane crops. These phytoplasmas were observed to be related to sugarcane white leaf phytoplasma that causes sugarcane disease in Asia [4]. Phytoplasma has also been reported to cause cynodon white leaf (CWL) disease in the Bermuda grass (Cynodon dactylon) [5], hyparrhenia white leaf disease (HWLD) in Hyparrhenia rufa grass [6], rice yellows dwarf disease (RYD) in rice [7] and sorghum grassy shoot (SGS) in sorghum crop plants (Sorghum stipoides) [4]. This is an indication that several other wild grass species could be infected by specific phytoplasma strains, hence; act as phytoplasma reservoirs which pose a threat to important feeds and food crops as well as reduce the forage supply of such wild grass strains for dairy farming.

The elimination of alternative phytoplasma hosts around napier farms as well as bioengineering of phytoplasma resistant variety of napier grass would constitute components for the management of phytoplasma diseases. This study identified phytoplasma wild host range among wild grasses and there genotypic distribution in western
Kenya necessary for the establishment of viable management and prevention strategies for the spread of Ns disease in napier grass and other important fodder.

**Materials and Methods**

**Sample collection**

Both phytoplasma symptomatic and asymptomatic wild grasses near Ns affected Napier fields from Bungoma and Busia counties were collected in this study (Table 1). Approximately 16 fields from each county were chosen from different sub-agro-ecological zones as replications. An itinerary for each area was set up. Fields in each area were chosen at random. In each field an average of 20 grass samples were obtained. The first samples were taken at the edge of every field which formed the base. Along transects placed 1-3m apart depending on the width of the field, one sample was collected per quadrat (1m x 1m) thrown 1-3m apart throughout the entire length of the transect. The numbers of plants were counted for the grasses. The collected samples were air-dried and transported to the international center for insect physiology and ecology (ICIPE-TOC) for further taxonomic identification, laboratory screening and phytoplasma diagnosis.

| County | Location | Samples |
|--------|----------|---------|
| Bungoma | Bungoma Township | 22 |
| Mlaha 1 | 18 |
| Mlaha 2 | 20 |
| Kibabii 1 | 22 |
| Kibabii 2 | 20 |
| Bisunu | 21 |
| Lwandanyi 1 | 20 |
| Lwandanyi 2 | 20 |
| Luya | 20 |
| Milo | 20 |
| Chetambe | 20 |
| Kimilii | 20 |
| Kibabii 3 | 19 |
| Kimaeli | 20 |
| Kokare | 20 |
| Katawa | 20 |
| Netima | 20 |
| Busia | Wakhungu-Odiado | 21 |
| Bumala | 20 |
| Bukhayo East | 20 |
| Otomong’ | 21 |
| Nambale | 21 |
| Elugulu | 20 |

**Table 1: Total number of grass samples collected for this study.**

**DNA extraction and PCR amplification**

Total DNA was extracted from 300 mg of leaf tissues by CTAB (cetyl trimethyl-ammonium bromide) method [8] and modified as described by Khan et al. [9]. DNA pellets were suspended in 50 μL deionized distilled water and the DNA suspensions stored at - 20°C.

PCR assays to amplify the phytoplasma DNA were performed using universal primer pairs P1/P6 [10] and NapF/NapR. The reaction mixture in the initial PCR contained 1.0 μL of template DNA of each sample at 100 ng/μL, 1.0 μL of both P1 and P6 primers for each sample, 2.5 μL of dNTPs (deoxyribonucleotide triphosphates) for each sample, 0.25 μL of Taq DNA polymerase (GenScript) for each sample and 2.5 μL of 1x Taq Polymerase buffer (GenScript) for each sample used. The PCR reaction mixture was gently vortexed for 10 seconds to mix and 22.25 μL of the mixture added to PCR tubes containing 1.0 μL of each template. A 35 cycle PCR was conducted using P1/P6 primer pair in a PTC-100® Thermal cycler (MJ Research, Incorporated, Lincoln Street, Massachusetts, USA) as follows; denaturation of DNA at 94°C for 2 minutes for 1 cycle, annealing of the primers at 52°C for 2 minutes for the first reaction and 72°C for 3 minutes for the subsequent reactions and elongation reaction at 72°C for 10 minutes for 1 cycle [11-13]. The second amplification of the primary PCR products of the 16S rDNA fragment was carried out using a reaction mixture containing NapF/NapR primer pair (Inqaba BiotecTM). The second round reaction was performed using 0.5 μL of the first PCR amplicons. From each of the second PCR amplicons, 6.0 μL of the DNA was mixed with 4.0 μL of 6X loading dye (SIGMA-ALDRICH®) prior to loading into the gel wells. Electrophoresis was carried out at 70 volts for 30 minutes on 1% (w/v) agarose gel containing 0.3 μg/mL ethidium bromide in 1x TAE (22.5mM Tris-acetate 1mM EDTA; pH 8.0) buffer. The gels were observed under UV transilluminator at 312nm wavelength to visualize the bands.

**DNA Purification and Sequencing of polymerase chain reaction (PCR) products**

The expected ≤ 800 bp nested polymerase chain reaction (nPCR) products obtained from the positive grass samples were purified on GenScript Quick Clean II PCR Extraction kit (GenScript’ Centennial Ave, Piscataway Township, NJ 08854, USA) as per the manufacturer’s protocol and directly sequenced. A total of 81 DNA amplicons that tested positive for phytoplasma were run on a gel at 1 μL (Figure 1) out
of which 33 representative samples (Table 2) were submitted for sequencing. To avoid redundancy, representative samples were selected based on the species of the grasses as well as the location the samples were obtained from. Sequencing was carried out in both directions (Forward and Reverse) using BigDye Terminator Cycle Sequencing in a DNA automated sequencer (SegoliLab, International Livestock Research Institute - ILRI, Nairobi Kenya).

**Results**

**Sequence homology and phylogenetic relationships**

The partial 16S rRNA genome sequences were assembled and edited using BioEdit sequence alignment editor [14]; gaps and ambiguities were eliminated from the final sequences. Partial full-length 16S rRNA gene sequences were converted to MEGA files for phylogenetic analysis by DNA neighbor-joining method using MEGA version 5.05 software [11] and the phylogenetic tree constructed with 1,000 bootstraps replications.

The 16S rRNA gene sequences of 33 phytoplasmas isolated from the wild grasses in this study were compiled in FASTA format and compared with each other and with 16 other reference phytoplasmas from NCBI Genbank database (appendix).

All phytoplasma sequences analyzed in this study aligned themselves in two discrete clades when compared to each other as depicted by phylogenetic tree (Figure 2). This study did not identify any novel phytoplasma strain (16S rRNA group/subgroup) from all the sequences characterized since the divergence of all the phytoplasma sequences retrieved in this study (Table 2) was below the recommended threshold of 97.5% sequence similarity (divergence of less than 2.5%) used in defining a novel phytoplasma species falling within the provisional status ‘Candidatus’ as per the International Research Program on Comparative Mycoplasmology [15]. This study, however, did not employ the use of 16S-23S rDNA spacer region in characterizing the phytoplasmas detected.

**Figure 1: Gel electrophoresis results of 1.0μL purified products obtained for direct sequencing.**

| Sample No. | Host Grass | County | Location | 16S rRNA Group | Partial sequence |
|------------|------------|--------|----------|----------------|-----------------|
| 46         | C. dactylon| Bungoma| Mlaha2    | 16SrXIV        | 16S rRNA gene   |
| 96         | P. maximum| Bungoma| Kibabii2  | 16SrXI         | 16S rRNA gene   |
| 97         | B. brizantha| Bungoma| Kibabii2  | 16SrXIV        | 16S rRNA gene   |
| 102        | B. brizantha| Bungoma| Bisunu    | 16SrXIV        | 16S rRNA gene   |
| 123        | C. dactylon| Bungoma| Bisunu    | 16SrXIV        | 16S rRNA gene   |
| 134        | C. dactylon| Bungoma| Lwandanyi1| 16SrXIV        | 16S rRNA gene   |
| 136        | C. dactylon| Bungoma| Lwandanyi1| 16SrXIV        | 16S rRNA gene   |
| 138        | C. dactylon| Bungoma| Lwandanyi2| 16SrXIV        | 16S rRNA gene   |
| 155        | C. dactylon| Bungoma| Lwandanyi2| 16SrXIV        | 16S rRNA gene   |
| 157        | C. dactylon| Bungoma| Lwandanyi2| 16SrXIV        | 16S rRNA gene   |
| 169        | B. brizantha| Bungoma| Luhya     | 16SrXIV        | 16S rRNA gene   |
| 266        | Other      | Bungoma| Kimaeli   | 16SrXI         | 16S rRNA gene   |
| 282        | B. brizantha| Bungoma| Kimaeli   | 16SrXI         | 16S rRNA gene   |
| 365        | B. brizantha| Busia | Bumala    | 16SrXI         | 16S rRNA gene   |
| 366        | Other      | Busia | Bumala    | 16SrXI         | 16S rRNA gene   |
| 413        | D. scalarum| Busia | Otimong’  | 16SrXI         | 16S rRNA gene   |
| 416        | C. dactylon| Busia | Otimong’  | 16SrXIV        | 16S rRNA gene   |
Table 2: Phytoplasma isolates, location of collection, host plant, and associated 16Sr groups retrieved in this study.

| ISOLATE | HOST PLANT | LOCATION | 16Sr GROUP |
|---------|------------|----------|------------|
| 418     | Poverty grass | Busia Otimong’ | 16SrXI |
| 478     | C. dactylon | Busia Marachi | 16SrXI |
| 479     | B. brizantha | Busia Marachi | 16SrXI |
| 484     | Poverty grass | Busia Bukhavoy west | 16SrXI |
| 491     | C. dactylon | Busia Bukhavoy west | 16SrXI |
| 515     | D. scalarum | Busia Bukhavoy central | 16SrXI |
| 519     | P. maximum | Busia Bukhavoy central | 16SrXI |
| 523     | D. scalarum | Busia Bukhavoy central | 16SrXI |
| 524     | B. brizantha | Busia Bukhavoy central | 16SrXI |
| 530     | D. scalarum | Busia Bwamani | 16SrXI |
| 579     | Poverty grass | Busia Bulanda2 | 16SrXI |
| 588     | E. indica | Busia Busia Township, ADC | 16SrXI |
| 593     | D. scalarum | Busia Busia Township, ADC | 16SrXI |
| 642     | P. maximum | Busia Busia Township, BP | 16SrXI |

Since this is a less significant taxonomic tool as compared to the 16S rDNA sequence [12]. It is recommended by IRPCM that phytoplasmas which differ with less than 2.5% of 16S rDNA nucleotide positions should be regarded as putative species when characterization is supported by data based upon molecular markers such as plant host range, insect vector transmission and serological studies rather than on 16S rDNA sequence [12].

**Figure 2:** An illustration of PCR products gel photograph highlighting the 1 kb DNA Ladder (M), phytoplasma positive Napier grass sample (N), the phytoplasma positive control (+), negative control (−), phytoplasma negative (lanes 146–152) and positive samples (lanes 156–163).

**Figure 3:** An unrooted bootstrap consensus tree constructed by the neighbor-joining analysis (MEGA, version 5.05) illustrating the phylogenetic relationships for the 16S rRNA genes of the phytoplasma strains derived from wild grasses in western Kenya. Notice the two distinct clades representing two different groups of phytoplasmas.
The NapF/NapR reactions from samples 46 (C. dactylon), 97 (B. brizantha), 102 (B. brizantha), 123 (C. dactylon), 134 (C. dactylon), 136 (C. dactylon), 139 (C. dactylon), 155 (C. dactylon), 157 (C. dactylon), 169 (B. brizantha), 416 (C. dactylon) yielded the expected 800 bp amplicons. Multiple sequence alignment via MEGA software version 5.05 showed that the above sequences were 99% identity with each other (Figure 2). BLAST (basic local alignment search tool) search program (www.ncbi.nih.gov/BLAST) showed that the above sequences were 99% similar to ‘Ca. Phytoplasma cynodontis’ (accession no. EU999999.1) and ‘Ca. Phytoplasma cynodontis’ (accession no. FJ348654.1). There was also 98% similarity with Bermuda grass white leaf isolates BGWL 1SL and PG as well as other several phytoplasma strains in group 16SrXIV from the NCBI database (Figure 3). BGWL disease was first reported in Bermuda grass (C. dactylon) in Kenya in 2010 [13].

On the other hand, multiple sequence alignment using MEGA software version 5.05 of the partial 16S rRNA gene sequences for the samples 479 (B. brizantha), 524 (B. brizantha), 366 (Other), 418 (Poverty grass), 365 (B. brizantha), 478 (C. dactylon), 579 (Poverty grass), 490 (D. scalarum), 523 (D. scalarum), 491 (C. dactylon), 515 (D. scalarum), 413 (D. scalarum), 530 (D. scalarum), 642 (P. maximum), 484 (Poverty grass), 266 (Other), 519 (P. maximum), 282 (B. brizantha), 588 (E. indica), 96 (P. maximum) and 593 (D. scalarum) showed 99% identity with each other (Figure 3).

Figure 4: A dendogram constructed by Neighbour-joining analysis (MEGA, version 5.05) of partial 16S rRNA gene sequences from 33 wild grass phytoplasmas from western Kenya in comparison to 16 other Ca. phytoplasma 16Sr group representatives from the NCBI GenBank. GenBank accession numbers are shown alongside. The evolutionary distances were computed using Maximum Composite Likelihood method and the bootstraps replicated 1000 times. The isolates from western Kenya are shown in sample numbers.

A BLAST search carried out on the above sequences revealed that there was 98-100% sequence similarity with the Ns (Napier grass stunt) phytoplasma isolate Mbita 2 (accession no. FJ862999.2) and Napier grass stunt phytoplasma isolate Bungoma (accession no. FJ862998.2) from Kenya, all of which belong to the phytoplasma group ‘Ca. Phytoplasma oryzae’ (group 16SrXI), as depicted in the phylogenetic tree (Figure 4).

Association between phytoplasma infection and grass species

From the 81 phytoplasma infections registered in this study (Table 3), C. dactylon had the highest proportion of total infections at 38%, followed by D. scalarum at 17.3%, B. brizantha had 16%, poverty grass and P. maximum had 7.4% and 4.9% respectively while E. indica and C. ciliaris had the least proportions of phytoplasma infection at 2.5% and 1.2% respectively.

| Grass species                  | PCR status | Proportion of infection | Total |
|--------------------------------|------------|------------------------|-------|
|                                | 0          | 1                      |       |
| Brachiaria brizantha           | 71(0.8452) | 13(0.1548)             | 16(1.0000) 84 |
| Cenchrus ciliaris              | 0          | 1(0.0000)              | 1(0.0000)  1 |
| Cymbopogon nardus              | 2(0.0000)  | 0                      | 2     |
| Cynodon dactylon               | 55(0.6395) | 31(0.3605)             | 86    |
| Digitaria scalarum             | 286(0.9533)| 14(0.0467)             | 14(0.17300) 300 |
| Echinichloa pyramidalis        | 2(1.0000)  | 0                      | 2     |
| Eleusine indica                | 6(0.7500)  | 2(0.2500)              | 8     |
| Eragrostis curvula             | 4(1.0000)  | 0                      | 4     |
| Hyparrhenia pilgerana          | 6(1.0000)  | 0                      | 6     |
| Other                          | 65(0.8784) | 9(0.1216)              | 74    |
| Panicum maximum                | 28(0.8750) | 4(0.1250)              | 32    |
| Pennisetum polystachion        | 5(1.0000)  | 0                      | 5     |
| Pennisetum purpureum           | 1(1.0000)  | 0                      | 1     |
| Poverty grass                  | 24(0.8000) | 6(0.2000)              | 6(0.7400)  30 |
| R. cochinchnensis              | 1(1.0000)  | 0                      | 1     |
| Setaria increassata            | 20(0.6667) | 1(0.3333)              | 3     |
| Sorghum versicolor             | 2(1.0000)  | 0                      | 2     |
| Sporobolus pyramidalis         | 4(1.0000)  | 0                      | 4     |
| Themeda triada                 | 1(1.0000)  | 0                      | 1     |
| Total                          | 565        | 81                     | 81(100)  646 |
| Chi square test                | 75.787(a)  | df                     |       |
| df                             | 18         |                        |       |
| Likelihood Ratio               | 68.054     |                        |       |
| P Value (≤0.05)                | 0.0001     |                        |       |

Table 3: Total grass species, their phytoplasma statuses and the proportions of infection.
Other grasses that were not identified constituted 11.1%. The proportions of phytoplasma infection per grass species were compared using two-sided Chi-Square tests at 95% confidence interval as summarized in the Table 2. From the test carried out, there was a strong association between proportions of phytoplasma infections and grass species (p = 0.0001).

### Association between 16S rRNA sub-group and grass species

Of all the grass samples that had positive phytoplasma infections, 33 were chosen for sequencing and phylogenetic analyses (Table 2). Two wild grass species that registered positive phytoplasma infections; *B. brizantha* and *C. dactylon* were infected by both phytoplasma subgroup 16SrXIV and 16SrXI. *B. brizantha* had 16SrXI: 16SrXIV infection ratio of 4:3 while *C. dactylon* had 16SrXI: 16SrXIV infection ratio of 2:9 (Figure 5). The remaining grass species positive for phytoplasma were entirely infected by phytoplasma subgroup 16SrXI (Table 4).

| Grass species         | 16SrXI | 16SrXIV | Not done | Total |
|-----------------------|--------|---------|----------|-------|
| *B. brizantha*        | 4(57.14%) | 3(42.86%) | 77       | 84    |
| *C. ciliaris*         | 0      | 0       | 1        | 1     |
| *C. nardus*           | 0      | 0       | 2        | 2     |
| *C. dactylon*         | 2(18.18%) | 9(81.81%) | 75       | 86    |
| *D. scalarum*         | 6(100%) | 0       | 294      | 300   |
| *E. pyramidalis*      | 0      | 0       | 2        | 2     |
| *E. indica*           | 1(100%) | 0       | 7        | 8     |
| *E. curvula*          | 0      | 0       | 4        | 4     |
| *H. pilgerama*        | 0      | 0       | 6        | 6     |
| *Other*               | 2(100%) | 0       | 72       | 74    |
| *P. maximum*          | 3(100%) | 0       | 29       | 32    |
| *P. polystachion*     | 0      | 0       | 5        | 5     |
| *P. purpureum*        | 0      | 0       | 1        | 1     |
| Poverty grass         | 3(100%) | 0       | 27       | 30    |
| *R. cochinchinensis*  | 0      | 0       | 1        | 1     |
| *S. incrassata*       | 0      | 0       | 3        | 3     |
| *S. versicolor*       | 0      | 0       | 2        | 2     |
| *S. pyramidalis*      | 0      | 0       | 4        | 4     |
| *T. triada*           | 0      | 0       | 1        | 1     |
| Total                 | 21     | 12      | 613      | 646   |

**Table 4:** A table of grass species collected and their associated 16S rRNA sub-group infections in different grass species collected.

### Discussion and Conclusions

This study found out that there was a strong association between proportions of phytoplasma infection and the grass species collected. *C. dactylon, B. brizantha, D. scalarum, P. maximum* and poverty grass generally act as wild phytoplasma hosts and are abundantly distributed in western Kenya. *E. indica* and *C. ciliaris* are scarcely distributed in western Kenya even though they play host to phytoplasma. There were substantial differences in proportions of phytoplasma infection per location of survey. There seems to be a trend in phytoplasma genotypic distribution in this study. The observed ecological isolation could be as a result of exclusive association of particular phytoplasmas with particular grass plant and/or insect host range in particular geographical regions. Gundersen et al observed that, two or more phytoplasma strains could exhibit specificity for preferred host plant in specific locations that may, to a large extent, reflect transmitting insect (vector) feeding behavior (Gundersen et al, 1996). This natural phytoplasmal ecological diversity may be exploited in the investigation of the epidemiology of phytoplasma-related diseases, hence the prevention of the spread of phytoplasma diseases. This was, however not verified as insect vectors were not collected and determined for correlation analysis in this study.

Phytoplasma subgroups 16SrXI and 16SrXIV were the only phytoplasma genotypes distributed among wild grasses in western Kenya. ‘Ca. Phytoplasma cynodontis’ (subgroup 16SrXIV): the causative agent of Bermuda grass white leaf disease (BGWLD) predominantly infects only *Cynodon dactylon* and *Brachiaria brizantha* wild grass types. This concurs with the findings made by Marcone et al where an association was made between BGWLD in *C. dactylon* (Bermuda grass) and ‘Ca phytoplasma cynodontis’, as well as *Brachiaria white leaf disease* [12]. Marcone et al demonstrated that phytoplasmas associated with Brachiaria white leaf disease and carpet grass white leaf showed 16S rDNA sequences identical or nearly similar to those of Bermuda grass white leaf in *C. dactylon*. On the other hand ‘Ca Phytoplasma oryzae’ (subgroup 16SrXI): the causative agent of Ns disease exhibited a broad pathogenic potential in this study and infects a large number of wild grasses, most importantly; *P. maximum, D. scalarum, poverty grass and B. brizantha.*

**Figure 5:** Percentages of phytoplasma 16S rRNA sub-group infections in different grass species collected.
### References

1. Jones P, Devonshire BJ, Holman TJ, Ajanga S (2004) Napier grass stunt: a new disease associated with a 16SrXI Group phytoplasma in Kenya. *Insect Science and its Application*, 21: 375-380.
2. Khan ZR, Pickett JA, Wadhams LJ, Muyekho F (2001) Habitat management strategies for the control of cereal stem borers and striga in maize in Kenya. *Insect Science and its Application*, 21: 375-380.
3. Mulaa MA, Muyekho F, Ajanga S, Jones P, Boa E (2004) Napier stunt disease vector identification and containment of the disease on farm. *Kenya Agricultural Research Institute (KARI)*.
4. Blanche KR, Tran-Nguyen LT, Gibb KS (2003) Detection, identification and significance of phytoplasmas in grasses in northern Australia. *Plant Pathology* 52: 505-512.
5. Salehi M, Izadpanah K, Siampour M, Taghizadeh M (2009) Molecular characterization and transmission of bermuda grass white leaf phytoplasma in Iran. *Journal of Plant Pathology*, 91: 655-661.
6. Obura E, Masiga D, Midega CAO, Otim M, Wachira F, et al. (2011) Hyparrhenia grass white leaf disease, associated with 16SrXI phytoplasma. *New Disease Reports*.
7. Lee LM, Pastore M, Vihio M, Danielli A, Attathom S, et al. (1997) Detection and Characterization of a Phytoplasma associated with annual Blue Grass (Poa annua) white leaf disease in Southern Italy. *European Journal of Plant Pathology* 103: 251-254.
8. Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.
9. Khan S, Qureshi MI, Kamaluddin Alam T, Abdin MZ (2006) Protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion. *African Journal of Biotechnology* 6: 175-178.
10. Deng S, Hiruki C (1991) Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. *Journal of microbiological methods* 14: 53-61.
11. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596-1599.
12. Marcone C, Schneider B, Seemuller E (2004) 'Candidatus phytoplasma cynodontis', the phytoplasma associated with Bermuda grass white leaf disease. International journal of Systematic and Evolutionary Microbiology 54: 1077-1082.

13. Obura E, Masiga D, Midega CAO, Wachira F, Pickett JA, et al. (2010) First report of a phytoplasma associated with bermuda grass white leaf disease in Kenya. New Disease Reports 21, 23.

14. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp Ser 41: 95-98.

15. IRPCM (International Research Program on Comparative Mycoplasmology) Phytoplasma/ Spiroplasma Working Team–Phytoplasma Taxonomy Group. (2004). 'Candidatus Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. International Journal of Systematic and Evolutionary Microbiology, 54: 1243-125.

16. Raj SK, Snehi SK, Khan MS, Kumar S (2008) 'Candidatus Phytoplasma asteris' (group 16SrI) associated with a witches'-broom disease of Cannabis sativa in India. Plant pathology 57: 1173.

17. Obura E, Midega CA, Masiga D, Pickett JA, Hassan M, et al. (2009) Recilia banda Kramer (Hemiptera: Cicadellidae), a vector of Napier stunt phytoplasma in Kenya. Naturwissenschaften 96: 1169-1176.

18. Li Z, Wu K, Zheng X, Zhang C, Wu Y, et al. (2008) First report of Bermuda grass white leaf phytoplasma infecting Cynodon dactylon in China. Unpublished.

19. Trinh HX, Ngo BG, Mai QV, Nguyen TD, Dang TV, et al. (2011) Diversity of phytoplasmas in sugarcane and rice in Vietnam. Unpublished.

20. Natsuki T, Al-Zadjali AD (2007) Detection, identification and molecular characterization of Candidatus Phytoplasma aurantifolia isolates associated with lime plants (Citrus aurantifolia) and other citrus species. Unpublished.

21. Davies RE (2011) 'Candidatus Phytoplasma pruni', a novel taxon associated with X-disease of stone fruits, Prunus spp.: multilocus characterization based on 16S rRNA, secY, and ribosomal protein genes. Unpublished.

22. Ember I, Acs Z, Munyanzeja JE, Crosslin JM, Kolber M (2011) Survey and molecular detection of phytoplasmas associated with potato in Romania and southern Russia. European Journal of Plant Pathology 130: 367-377.

23. Arocha-Ro rate Y (2011) Identification of Grominella nigrifrons as a potential vector for phytoplasmas identified in Canada. Unpublished.

24. Priy M, Chaturvedi Y, Rao GP, Raj SK (2010) First report of phytoplasma 'Candidatus Phytoplasma trifolii' (16SrVI) group associated with leaf yellows of Calotropis gigantea in India. New Disease Reports 22: 29.

25. Lee IM, Bottner-Parker KD, Zhao Y, Bertaccini A, Davis RE (2012) Differentiation and classification of phytoplasmas in the pigeon pea witches'-broom group (16SrIX): an update based on multiple gene sequence analysis. Int J Syst Evol Microbiol. In press.

26. Sousa E, Marques A, Cardoso F (2011) The first report of Candidatus phytoplasma pyri in the pear-leafhopper Pyylla pyri in Portugal. Unpublished.

27. Daily EL, Bottner KD, Davis RE (2000) Revised Subgroup Classification of Group V Phytoplasmas and Placement of Flavescence Doree-Associated Phytoplasmas in Two Distinct Subgroups. Unpublished.

28. Rao GP, Mall S, Marcone C (2008) First report of Bermuda grass white leaf phytoplasma infecting Dicentham annulatum grass in India. Unpublished.

29. Balakhishieva G, Qurbanov M, Mammadov A, Bayramov S, Foisac X (2010) Identification of ‘Ca. P. brasilienise’ from a yellowing peach tree in Azerbaijan and development of a specific detection test. Unpublished.