Journal of
Plant Pathology & Microbiology

Adam, et al., J Plant Pathol Microb 2015, 6:6
DOI: 10.4172/2157-7471.1000274

Research Article Open Access

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

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Rec date: Feb 16, 2015; **Acc date:** June 22, 2015; **Pub date:** June 24, 2015

Abstract

Napier grass (*Pennisetum purpuruem*) 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 crossectional 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 purpuruem*) 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 km^2 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 stipoideum*) [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
	Kimilili	20
	Kibabii 3	19
	Kimaeti	20
	Kokare	20
	Katakwa	20
	Netima	20
Busia	Wakhungu-Odiado	21
	Bumala	20
	Bukhayo East	20
	Otimong'	21
	Nambale	21
	Elugulu	20
	Marachi	20
	Bukhayo West	20
	Bukhayo Central	20
	Bwamani	21
	Bulanda 1	20
	Bulanda 2	20
	Busia Township, ADC	20
	Aget	20
	Busia Township, BP	20
TOTAL		646

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).

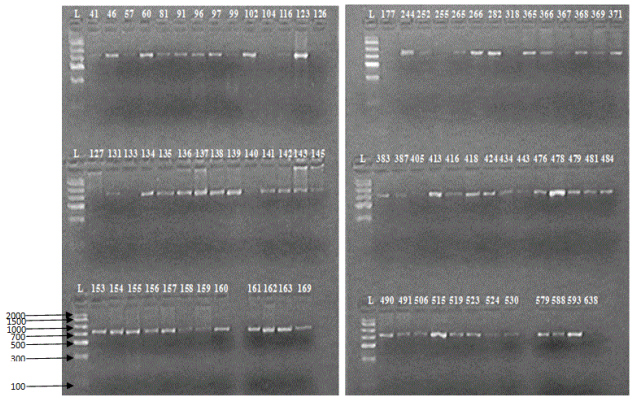

Figure 1: Gel electrophoresis results of 1.0µL purified products obtained for direct sequencing.

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.

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	Lwandanyi1	16SrXIV	16S rRNA gene
139	*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	Kimaeti	16SrXI	16S rRNA gene
282	*B. brizantha*	Bungoma	Kimaeti	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

418	Poverty grass	Busia	Otimong'	16SrXI	16S rRNA gene
478	*C. dactylon*	Busia	Marachi	16SrXI	16S rRNA gene
479	*B. brizantha*	Busia	Marachi	16SrXI	16S rRNA gene
484	Poverty grass	Busia	Marachi	16SrXI	16S rRNA gene
490	*D. scalarum*	Busia	Bukhayo west	16SrXI	16S rRNA gene
491	*C. dactylon*	Busia	Bukhayo west	16SrXI	16S rRNA gene
515	*D. scalarum*	Busia	Bukhayo central	16SrXI	16S rRNA gene
519	*P. maximum*	Busia	Bukhayo central	16SrXI	16S rRNA gene
523	*D. scalarum*	Busia	Bukhayo central	16SrXI	16S rRNA gene
524	*B. brizantha*	Busia	Bukhayo central	16SrXI	16S rRNA gene
530	*D. scalarum*	Busia	Bwamani	16SrXI	16S rRNA gene
579	Poverty grass	Busia	Bulanda2	16SrXI	16S rRNA gene
588	*E. indica*	Busia	Busia Township, ADC	16SrXI	16S rRNA gene
593	*D. scalarum*	Busia	Busia Township, ADC	16SrXI	16S rRNA gene
642	*P. maximum*	Busia	Busia Township, BP	16SrXI	16S rRNA gene

Table 2: Phytoplasma isolates, location of collection, host plant, and associated 16Sr groups retrieved in this study.

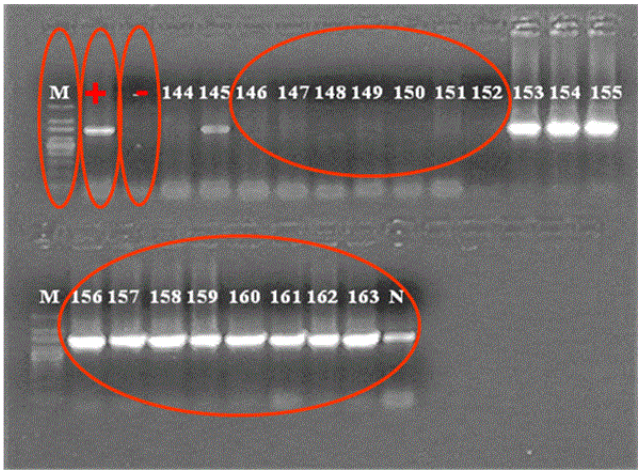

Figure 2: An illustration of PCR products gel photograph highlighting the 1 kb DNA Ladder (M), phytoplasma positive Napier grass sample (N), the phytoplasma positive control (+), negative control (–), phytoplasma negative (lanes 146–152) and positive samples (lanes 156–163).

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].

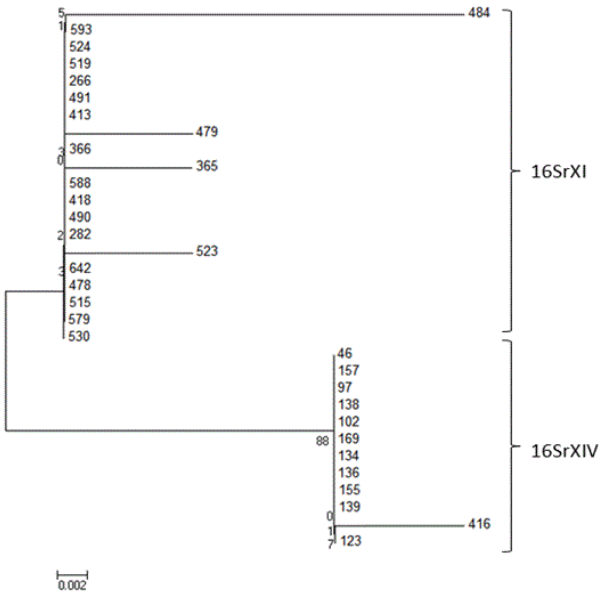

Figure 3: An unrooted bootstrap consensus tree constructed by the neighbor-joining analysis (MEGA, version 5.05) illustrating the phylogenetic relationships for the 16S rRNA genes of the phytoplasma strains derived from wild grasses in western Kenya. Notice the two distinct clades representing two different 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*), 138(*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 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).

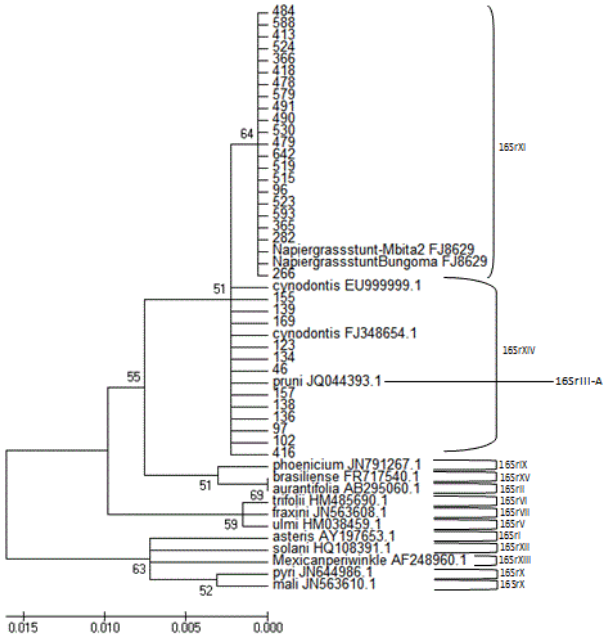

Figure 4: A dendogram constructed by Neighbour-joining analysis (MEGA, version 5.05) of partial 16S rRNA gene sequences from 33 wild grass phytoplasmas from western Kenya in comparison to 16 other Ca. phytoplasma 16Sr group representatives from the NCBI GenBank. GenBank accession numbers are shown alongside. 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)	13(16.0000)	84
Cenchrus ciliaris	0	1(1.0000)	1(1.2000)	1
Cymbopogon nardus	2(1.0000)	0	0	2
Cynodon dactylon	55(0.6395)	31(0.3605)	31(38.3000)	86
Digitaria scalarum	286(0.9533)	14(0.0467)	14(17.3000)	300
Echinichloa pyramidalis	2(1.0000)	0	0	2
Eleusine indica	6(0.7500)	2(0.2500)	2(2.5000)	8
Eragrostis curvula	4(1.0000)	0	0	4
Hyparrhenia pilgerama	6(1.0000)	0	0	6
Other	65(0.8784)	9(0.1216)	9(11.1000)	74
Panicum maximum	28(0.8750)	4(0.1250)	4(4.9000)	32
Pennisetum polystachion	5(1.0000)	0	0	5
Pennisetum purpureum	1(1.0000)	0	0	1
Poverty grass	24(0.8000)	6(0.2000)	6(7.4000)	30
R. cochinchinensis	1(1.0000)	0	0	1
Setaria incrassata	2(0.6667)	1(0.3333)	1(1.2000)	3
Sorghum versicolor	2(1.0000)	0	0	2
Sporobolus pyramidalis	4(1.0000)	0	0	4
Themeda triada	1(1.0000)	0	0	1
Total	565	81	81(100)	646
Chi square test	75.787(a)			
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.

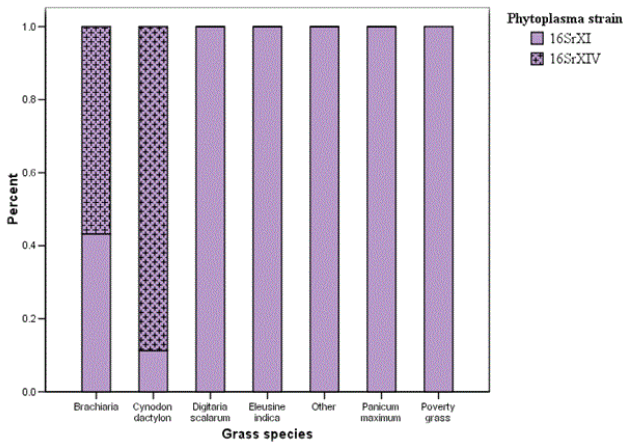

Figure 5: Percentages of phytoplasma 16S rRNA sub-group infections in different grass species collected.

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. datylon*. 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*.

Isolate	Acronyms	Phytoplasma species	16S rRNA Group-subgroup	Host species	Location	NCBI Accession No.	Literature
Aster yellows	MIAY	Ca. P *asteris*	16SrI	*Cannabis sativa L*	India	EU439257.1	[16]
Napier grass stunt	NSD	Ca. P *oryzae*	16SrXI	*P. purpuruem*	Kenya, Mbita	FJ862999.2	[17]
Napier grass stunt	NSD	Ca. P *oryzae*	16SrXI	*P. purpuruem*	Kenya, Bungoma	FJ862997.2	[17]
Bermuda grass white leaf	BGWL	Ca. P *cynodontis*	16SrXIV	*Cynodon dactylon*	China	EU999999.1	[18]
Rice yellow Dwarf	RYD	Ca. P oryzae	16SrXI	*Oryza sativa*	Vietnam	JF927999.1	[19]
Peanut witches' - broom	PWB	Ca. P *aurantifolia*	16SrII	*Citrus araurantifolia*	Oman, Rumis	AB295060.1	[20]
X-disease	PX11Ct1	Ca. P *pruni*	16SrIII-A	*stone fruits, Prunus*	U.S.A/ canada	JQ044393.1	[21]
Stolbur	STOL	Ca. P *solani*	16SrXII-A	*Solanum tuberosum*	Romania/ Russia	HQ108391.1	[22]
Elm yellows	EY20_SRB	Ca. P *ulmi*	16SrV-A	*Ulmus spp*	Serbia	HM038459.1	[24]
Clover proliferation	CP	Ca. P *trifolii*	16SrVI	*Calotropis gigantean*	India: Gorakhpur	HM485690.1	[26]
Ash yellows	AY	Ca. P *fraxini*	16SrVIIA	*Graminella nigrifrons*	Canada	JN563608.1	[24]
Pigeonpea witches'-broom	PPWB	Ca. P *phoenicium*	16SrIX	*Blueberry*	U.S.A	JN791267.1	[25]
Apple proliferation	AP	Ca. P *mali*	Gn-16SrXA	*Graminella nigrifrons*	Canada	JN563610.1	[23]
Apple proliferation	AP	Ca. P *pyri*	16SrX	*Cacopsylla pyri*	Portugal	JN644986.1	[26]
Mexican periwinkle viresc	MPWV	Unidentified	16SrXIII-A	*Catharanthus roseus*	U.S.A	AF248960.1	[27]
Bermuda grass white leaf	BGWL	Ca. P *cynodontis*	16SrXIV	*Dicanthium annulatum*	India	FJ348654.1	[28]
Hibiscus witches'-broom	HWB	Ca. P *brasiliense*	16SrXV	*Prunus persica*	Azerbaijan	FR717540.1	[29]

Appendix: Acronyms and GenBank accession numbers of phytoplasma 16S rDNA sequences used for phylogenetic analysis.

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