Detection, Identification, and Significance of Phytoplasmas in Wild Grasses in East Africa

George O. Asudi, Unit for Environmental Sciences and Development, North-West University, Potchefstroom 2520, South Africa; International Centre of Insect Physiology and Ecology, P.O. Box 30772-0010 Nairobi, Kenya; Biochemistry and Biotechnology Department, Kenyatta University, P.O. Box 43844-00100, Nairobi, Kenya; **Johnnie Van den Berg,** Unit for Environmental Sciences and Development, North-West University, Potchefstroom 2520, South Africa; **Charles A. O. Midega,** International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya; **Bernd Schneider** and **Erich Seemüller,** Julius Kuhn Institute, Federal Research Centre for Cultivated Plants Institute for Plant Protection in Fruit Crops and Viticulture, 69221 Dossenheim, Germany; **John A. Pickett,** Biological Chemistry and Crop Protection Department Rothamsted Research, Harpenden, Herts, AL5 2JQ, UK; and **Zeyaur R. Khan,** Unit for Environmental Sciences and Development, North-West University, South Africa and ICIPE, Kenya

Abstract

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Plant-pathogenic phytoplasmas found in wild grasses in East Africa could pose a serious threat to the cultivation of Napier grass, *Pennisetum purpureum*, the most important livestock fodder in the region. To asses this threat, leaves from plants of 33 grass species were sampled from Mbita, Bungoma, and Busia districts in western Kenya; Tarime district in northern Tanzania; and Busia and Bugiri districts in the eastern Uganda to determine which species host phytoplasmas, the identity of the phytoplasmas, and their relationship with disease symptoms. Phytoplasmas were detected using universal primers based on conserved phytoplasma-specific 16S rDNA sequences from 11 grass species collected. Sequence and phylogenetic analysis revealed the presence of Napier grass stunt-related phytoplasmas in 11 grass species, '*Candidatus* Phytoplasma cynodontis' in three, and goosegrass white leaf phytoplasma in 2 wild grass species. This study showed that the geographical distribution, diversity of phytoplasmas, and their grass host species in East Africa is greater than antecedently thought and that typical disease symptoms, including white leaf or stunting alone, are not reliable indicators of the presence of phytoplasma. It also shows the need to identify insect vectors responsible for phytoplasma transmission from native grasses to Napier grass or other cereals present in the region.

Phytoplasmas are cell wall-less bacteria belonging to the class Mollicutes, order Acholeplasmatales, and the new taxon '*Candidatus* Phytoplasma' (IRPCM Phytoplasma/Spiroplasma working team—Phytoplasma Taxonomy Group 2004). Globally, phytoplasmas are associated with numerous plant diseases (Lee et al. 2000). The inability to isolate and culture phytoplasmas axenically prevented their taxonomic and systematic classification for a long time. Therefore, phytoplasmas were largely identified and classified based on symptoms, vector specificity, and host range (Bertaccini 2007; Lee et al. 2000). A general and reliable system of phytoplasma detection and taxonomic classification was developed based on molecular tools such as polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) of the conserved 16S ribosomal gene (16S rDNA). This system provided a rapid and reliable means for preliminary classification of phytoplasmas (Lee et al. 1998). Currently, 37 '*Ca.* P.' spp. have been formally described based on the similarity of their 16S rDNA sequences with new lists of species being documented regularly (Harrison et al. 2014; IRPCM Phytoplasma/Spiroplasma working team—Phytoplasma Taxonomy Group 2004; Quaglino et al. 2013; Win et al. 2013a).

In East Africa, the most important livestock fodder affected by phytoplasmas is Napier grass (*Pennisetum purpureum* Schumach). Two phytoplasmas have been identified in *P. purpureum*, one designated Napier grass stunt (NGS) and the other African sugarcane yellow leaf (ASYL) (Jones et al. 2004, 2007; Nielsen et al. 2007). These pathogens cause foliar yellowing, small leaves, proliferation of tillers, and shortening of internodes with severely stunted clumps in Napier grass. Often, the whole grass stool is affected, with a complete loss in yield leading to eventual death of the infected plant. The disease has negatively affected the livelihoods of farmers that rely on the crop as their main source of feed for dairy animals. In some fields, the disease has caused up to 100% loss of the crop, forcing the smallholder farmers to reduce the number of dairy cattle or purchase fodder from the local market (Orodho 2006). NGS belongs to the 16SrXI group, '*Ca.* P. oryzae', or rice yellow dwarf while ASYL belongs to the 16SrIII, '*Ca.* P. pruni', or X-disease group (Arocha et al. 2009; Jones et al. 2004, 2007; Nielsen et al. 2007; Obura et al. 2009). The leafhopper *Maiestas banda* (Kramer) (Hemiptera: Cicadellidae) transmits NGS in Napier grass in Kenya (Obura et al. 2009), while the leafhopper *Exitianus* spp. (Hemiptera: Cicadellidae) and planthopper *Leptodelphax dymas* (Fennah) (Hemiptera: Delphacidae) have been suggested as potential vectors in the transmission of ASYL (Arocha et al. 2009).

Two phytoplasmas closely related to the NGS group have been reported in other grasses in western Kenya. These phytoplasmas are Bermuda grass white leaf (BGWL), found in *Cynodon dactylon* (Obura et al. 2010), and Hyparrhenia grass white leaf (HGWL), found in *Hyparrhenia rufa* (Obura et al. 2011). HGWL is classified as a '*Ca.* P. oryzae' strain and is closely related to NGS, sharing a 99% 16S rDNA sequence identity, while BGWL displays a 100% identity with '*Ca* P. cynodontis' strain LY-C1. The potential phytoplasma host characteristics suggested that *H. rufa* could be an alternative host for NGS phytoplasma, and it could play an important role in the spread of this pathogen in East Africa (Obura et al. 2011).

The basic epidemiological cycle of phytoplasma diseases consists of three components: the phytoplasma, a susceptible host plant, and a suitable vector that feeds on the host plant (Lee et al. 2003). This three-way interaction plays a significant role in determining the spread of phytoplasmas (Lee et al. 2003; Obura et al. 2009). Accordingly, the analysis of epidemiological systems of phytoplasmas with many host plants needs to take into account the occurrence of other natural plant hosts and their role as alternative inoculum sources as well as the host range and feeding preferences of vector species or particular vector

Corresponding author: G. O. Asudi; E-mail: gasudii@gmail.com

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populations (Lee et al. 2003; Obura et al. 2011; Weintraub and Beanland 2006). By extending previous survey work, the present study sought to discover whether there were other phytoplasmas and host grass species with the potential to affect Napier grass and other monocots in Kenya and neighboring countries.

Materials and Methods

Study area. Wild grasses were collected from Bungoma and Busia districts located in the western part of Kenya, Tarime district in the northern part of Tanzania, and Busia and Bugiri districts in the eastern part of Uganda. Plant materials were collected between July 2012 and August 2013. The altitudes at surveyed areas ranged from 947 to 1,697 m above sea level (a.s.l) in Kenya, 1,079 to 1,227 m a.s.l. in Uganda, and 1,400 to 1,700 m a.s.l in Tanzania. Surveys were also conducted in and along protected areas of Ruma National Park in the Lambwe Valley in the Mbita district. Other wild grass samples were collected in a grass nursery maintained at the International Centre of Insect Physiology and Ecology (ICIPE) Thomas Odhiambo Campus located at Mbita point, Mbita district, western Kenya (Fig. 1).

Plant materials. Both asymptomatic and symptomatic wild grasses were collected from randomly selected fields bordering Napier grass farms in Bungoma and Busia districts of western Kenya, Tarime district of northern Tanzania, and Busia and Bugiri districts of eastern Uganda. In each of these fields, four quadrats (1 m by 1 m) were randomly thrown along demarcated transects and grass stands. Plant leaves were sampled from five different plants in each quadrat. Grass samples were randomly picked in and around the park whereas, in the nursery, all grass species were sampled. For symptomatic grasses, leaves were collected from plant species exhibiting at least one of the following symptoms: yellow, white, or creamy-colored leaves; abnormal tillers; stunting; or floral deformation. The grass species were collected with the inflorescence to facilitate species identification (Muyekho et al. 2004). After collection, all plant materials were transported and stored at −20°C before isolating DNA. Collection sites were mapped using the Global Positioning System to provide accurate information on the locations.

Phytoplasma DNA isolation and amplification. DNA was extracted from leaves as described previously (Obura et al. 2009). The DNA pellet was air dried at room temperature and reconstituted in 50 μl of sterile water. The quality of isolated DNA was determined using 1% (wt/vol) agarose gel and quantified using a spectrophotometer (Jenway Genova Plus Spectrophotometer). DNA samples were stored at −20°C until further use. Phytoplasma DNA was amplified using universal primer pair P1/P6 (Deng and Hiruki 1991) in the first-round PCR followed by primer pair NapF/NapR (Obura 2012) in a PTC-100 Thermal cycler (MJ Research) or Proflex PCR machine (Applied Biosystems). Amplicons were then visualized by gel electrophoresis in a 1% agarose gel stained with ethidium bromide using 1× Tris-acetate EDTA (40 mM Tris acetate and 1 mM EDTA, pH8.0) as running buffer, and photographed. In all the experiments, water controls were included in which no plant nucleic acid was added to the PCR reaction mix as negative controls. DNA from the reference phytoplasma strain, NGS, maintained at ICIPE Mbita station was used as a positive control.

Phytoplasma DNA was also amplified using the P1/Tint primer pair (Smart et al. 1996) using a Bio-Rad iCycler (Germany). The PCR products were directly purified using the Qiaquick purification kit (Qiagen Inc.) following the manufacturer's instructions.

Sequence and phylogenetic analysis. The 16S rDNA amplicons of phytoplasma-positive grasses were purified using a QuickClean II PCR Extraction Kit (GenScript, USA Inc.) according to the manufacturer's instructions. DNA amplicons were sequenced directly using the NapF and NapR primers using Dye Terminator chemistry in a DNA automatic sequencer at the International Livestock Research Institute, Nairobi, Kenya. P1/Tint amplicons were ligated into pGEM-T easy vector system (Promega Corp.) and cloned using *Escherichia coli* NM522 strain (Promega Corp.). Successful clones were then purified using EasyPrepR Pro Plasmid Miniprep Kit (Biozym, Germany) according to the manufacturer's instructions. DNA sequences were assembled and edited using DNA Workbench (CLC bio, Aarhus, Denmark) software. The resulting consensus sequences are deposited in the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov/). For phylogenetic analysis, sequences of phytoplasma infecting grasses were obtained from the GenBank database

Fig. 1. Map of East Africa showing the locations of the study.

(Table 1). The 16S rDNA and the SR sequences were aligned using a progressive alignment algorithm (Feng and Doolittle 1987) implemented in the DNA Workbench package (CLC bio, QIAGEN, Aarhus) with the default settings. The alignments were then exported in fasta format, converted to MEGA 6 format, and used for distance and phylogenetic analyses in MEGA 6 software (Tamura et al. 2013). A dendrogram was then constructed with the neighbor-joining method (Saitou and Nei 1987) using 1,000 replicates for bootstrap analysis (Felsenstein 1985) (Fig. 2).

Results

Host plants. The grass species tested, collection sites, symptom description, number of samples, and phytoplasma presence are listed in Table 2. In total, 2,185 wild grass samples were collected, consisting of 830 from Bungoma and Busia districts of western Kenya, 400 from Tarime in northern Tanzania, 850 from Busia and Bugiri districts in eastern Uganda, and 105 wild grass samples from Mbita district. Samples from Mbita district comprised 65 grasses from the ICIPE grass nursery and 40 samples collected in Ruma National Park. Wild grasses collected in the five districts comprised 28 species in 21 genera while those from Mbita district comprised 33 species in 22 genera. Ninety-three grass samples could not be identified because they lacked inflorescences (Table 2). Phytoplasma infection was confirmed by the amplification of a 778-bp 16S rDNA fragment in nested PCR (Fig. 3) from 270 of 2,185 wild grass samples tested (Table 2). Of all the grass samples, 81 of 850 sampled in Kenya (Bungoma and Busia districts), 61 of 400 from Tarime district, and 74 of 830 of grasses from Uganda (Busia and Bugiri districts) were phytoplasma positive. Of 105 samples from Mbita district, 55 were positive for phytoplasma. In the Busia and Bungoma districts of Kenya, phytoplasmas were detected in 1 of 74 *Brachiara brizantha,* 2 of 47 *Eleusine indica*, 55 of 132 *C. dactylon*, 21 of 51 *H. rufa*, 1 of 33 *Sporobolus pyramidalis*, and 1 of 1 *Coix laryma-jobi* samples collected. In the Tarime district of Tanzania, phytoplasmas were detected in 6 of 40 *B. brizantha*, 41 of 107 *C. dactylon*, 1 of 12 *E. indica*, 9 of 24 *H. rufa*, and 2 of 13 *S. pyramidalis* plants. Phytoplasmas were also detected in five other grass species found in Uganda, consisting of 4 of 98 *B. brizantha*, 62 of 124 *C. dactylon*, 3 of 89 *Digitaria scalarum*, 2 of 29 *H. rufa*, and 3 of 82 *Sorghum versicolor* samples. In Mbita district, 1 of 4 *Chloris gayana*, 30 of 30 *C. dactylon*, 1 of 5 *D. scalarum*, 1 of 2 *Enteropogon macrostachyus*, 1 of 2 *H. cymbaria*, 15 of 20 *H. rufa*, and 2 of 6 *B. brizantha* plants tested positive for phytoplasma. Of the 198 *P. purpureum* samples collected, 45 of 91 from Kenya, 26 of 57 from Uganda, and 6 of 50 from Tanzania tested positive for phytoplasma (Table 2).

Relationship between phytoplasma detection and disease symptoms. Phytoplasma-infected Bermuda grass (*C. dactylon*) plants showed whitening of leaves, a bushy growing habit, small leaves, shortened stolons or rhizomes, stunting, and proliferation of auxiliary shoots. These symptoms were similar in all locations surveyed. Symptomatic thatch grasses (*H. rufa*) appeared stunted and bushy, with small white leaves. These symptomatic thatch grasses were mainly found in grass veld at several locations of Lambwe valley in western Kenya and in the wild grassland of the Nyamongo village in the Tarime district in northern Tanzania. Some of the infected *B. brizantha* and *S. pyramidalis* plants showed yellow leaf symptoms while the remaining phytoplasma-positive species did not show symptoms (Tables 2 and 3). Of the 11 wild grass species that tested positive for phytoplasma, 9 (i.e., *B. brizantha*, *Chloris gayana*, *C. laryma-jobi*, *Eleusine indica*, *D. scalarum*, *E. macrostachyus*, *H. cymbaria*, *S. pyramidalis*, and *S. versicolor*) are newly recorded phytoplasma hosts in the region. The *H. rufa* and *C. dactylon* plants that tested positive for phytoplasmas in Busia and Bugiri districts of Uganda and Tarime district of Tanzania are newly reported phytoplasma hosts in these areas.

The relationship between phytoplasma presence in leaves and symptoms varied according to plant species. There was a clear association between phytoplasma and white leaf symptoms in *C. dactylon*. All symptomatic *C. dactylon* plants were phytoplasma positive and asymptomatic plants were negative. There was also a clear association between a phytoplasma infection and stunting, bushy-growth habits, and white leaf symptoms in *H. rufa*. However, in *D. scalarum* and *Cyperus rotundus*, yellow leaf symptoms were not associated with phytoplasma presence. Some of the *B. brizantha* and *S. pyramidalis* plants had yellow leaf symptoms while the remaining phytoplasma-positive species were asymptomatic. Symptom descriptions are provided in Tables 2 and 3.

Genetic relatedness of phytoplasmas infecting wild grasses. Comparison of the sequences obtained indicated that phytoplasmas from the wild grass species were different. Thus, among the grasses collected in Kenya, phytoplasmas found in *C. gayana, C. laryma-jobi*, *D. scalarum*, *E. macrostachyus*, *E. indica*, *H. rufa*, *H. cymbaria*, and *S. pyramidalis* were closely related to the 16SrXI group of phytoplasmas. They shared sequence similarities that ranged from 98.43 to 98.95% with the 16SrXI phytoplasma sequences of NGS phytoplasmas reported previously in Uganda and Kenya (GenBank accession numbers AY377876, EF012650, FJ862997, FJ862998, FJ862999, JQ868440, and JQ868443). In *C. dactylon*, phytoplasmas from two 16S groups were found. One accession had the NGS phytoplasma strain while the remaining accessions had their highest sequence similarity with BGWL strains, which belong to the 16SrXIV group (accession numbers EU409293, AJ550984, AJ550986, AJ550985, EF444485, HE599391, HE599395, AF248961, AB052871, EU234510, AB642601, AB741630, HE599392, HE599393, and KF234570). The sequence similarity with reference strains ranged from 99.47

Table 1. Acronyms and National Center for Biotechnology Information accession numbers of phytoplasma 16S ribosomal DNA (rDNA) sequences used for phylogenetic analyses

Strain[a]	Phytoplasma	16S rDNA group	Host plant	Origin	EMBL accession[b]
BGWL	Bermuda grass white leaf	16SrXIV	Bermuda	India	KF234570
BGWL	Bermuda grass white leaf	16SrXIV	Bermuda	Turkey	HE599393
BGWL-LY-C1	Bermuda grass white leaf	16SrXIV	Bermuda	China	EU409293
GGWL	Goose-grass white leaf	-	Goose-grass	Myanmar	AB741629
SGSVarI	Sorghum grassy shoot VarI	16SrXI-B	Coast button grass	Australia	AF509324
SGSVarII	Sorghum grassy shoot VarII	16SrXI-B	Purpletop Rhodes	Australia	AF509325
NGS	Napier grass stunt	16SrXI	Napier	Kenya	JQ868443
NGS	Napier grass stunt	16SrXI	Napier	Kenya	JQ868440
NGS	Napier grass stunt	16SrXI	Napier	Kenya	FJ862999
NGS	Napier grass stunt	16SrXI	Napier	Kenya	FJ862998
NGS	Napier grass stunt	16SrXI	Napier	Kenya	FJ862997
NGS	Napier grass stunt	16SrXI	Napier	Uganda	EF012650
NGS	Napier grass stunt	16SrXI	Napier	Kenya	AY377876
NGS	Napier grass stunt	16SrIII	Napier	Ethiopia	DQ305977
BVK	'Psammotettix cephalotes' flower stunt	16SrXI-C	Periwinkle	Germany	HQ589192
SUK22-4	Sugarcane green grassy shoot phytoplasma	-	Sugarcane	Thailand	KF908793

[a] BGWL = Bermuda grass white leaf, GGWL = goose grass white leaf, SGS = sorghum grassy shoot, and NGS = Napier grass stunt.
[b] EMBL accession number.

to 99.6%. In *B. brizantha* plants sampled in Mbita district, three phytoplasma strains were detected. One strain had a close similarity with NGS (99%), the second belonged to BGWL (99.6%), and the third strain was identified as a goose-grass white leaf (GGWL) phytoplasma having a 99.6% sequence similarity to GenBank accession AB741629. All phytoplasmas were detected in separate plants.

In Tanzania, phytoplasmas closely related to BGWL strains were common and were detected in the majority of *C. dactylon* plants sampled. Only one sample contained a phytoplasma strain closely related to NGS. '*Ca.* P. cynodontis' was also detected in one *H. rufa* and one *E. indica* plant. These phytoplasmas were closely related to a BGWL strain from India, which belongs to the 16SrXIV group with a 99.6% sequence similarity. Two different phytoplasmas were

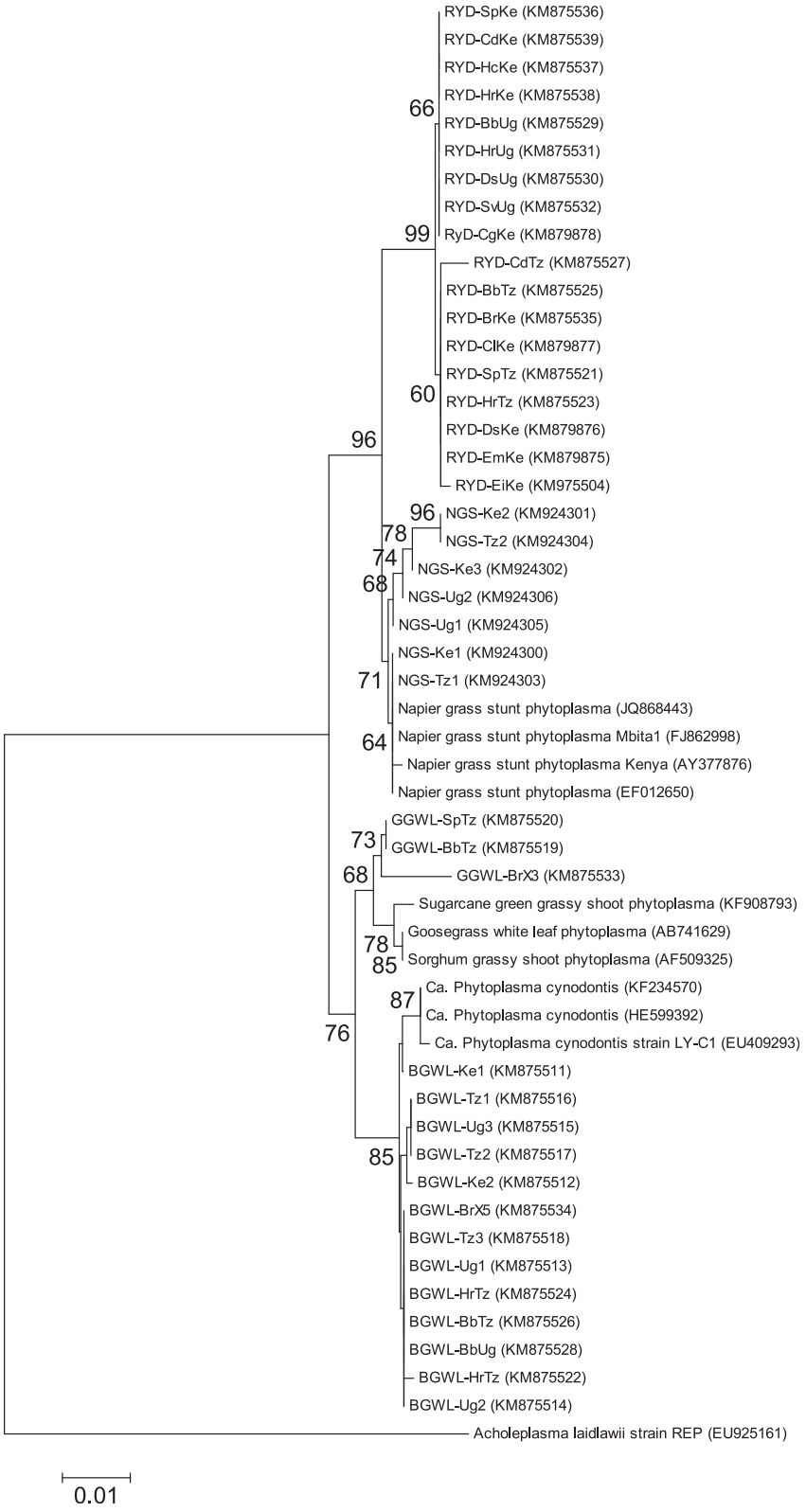

Fig. 2. Phylogenetic dendrogram of the 16S ribosomal RNA gene sequences of 48 phytoplasmas generated by Mega6, as described in the text. Bootstrap values were based on 1,000 replicates. Phytoplasma names and sequence accession numbers are provided in Tables 1 and 3.

detected in *S. pyramidalis* species collected in Tanzania. One had a close resemblance to GGWL (GenBank accession number AB741629) with 99.85% sequence similarity, while the other was similar to NGS isolates from Uganda and Kenya sharing a 98.83% similarity with sequences in databases (GenBank accession numbers AY377876, EF012650, FJ862997, FJ862998, FJ862999, JQ868440, and JQ868443). Eight of the nine phytoplasma-infected *H. rufa* plants collected from Tarime were classified as NGS phytoplasma with a 98.95% sequence similarity. In individual *B. brizantha* accessions, three different phytoplasmas were detected—namely,

Table 2. Grass species screened for the presence of phytoplasmas from East Africa

Grass species	Common name[a]	Location	Symptoms	Phytoplasma[b]	Positive/tested[c]
Andropogon gayanus	Gamba grass	Mbita	None	–	0/1
Bothriochloa bladhii	Bluestem	Mbita	None	–	0/1
B. insculpta	Sweet pitted grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	None	–	0/19
Brachiaria brizantha	Signal grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	Yellow leaves	+*	13/218
Brachiaria sp.	Signal grass	Bungoma, Busia-K, Tarime	None	–	0/126
Cenchrus ciliaris	African foxtail	Mbita	None	–	0/1
Chloris gayana	Rhodes grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	None	–	1/17
C. roxybarghiana	Horsetail grass	Bungoma, Busia-K, Tarime, Mbita	None	–	0/7
Coix laryma-jobi	Otiro (Luo)	Busia-K	None	+*	1/1
Cymbopogon citratus	Lemon grass	Mbita	None	–	0/2
C. nardus	Blue citronella	Bungoma, Busia-K, Tarime, Mbita	None	–	0/12
Cynodon dactylon	Star/bermuda grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	White leaves, stunting	+	192/393
Cyperus rotundus	Sedge	Bungoma, Busia-K, Tarime, Bugiri, Busia-U	Yellow leaves	–	0/123
Danthonia sp.	Poverty grass	Bungoma, Busia-K, Bugiri, Busia-U	None	–	0/33
Digitaria scalarum	Couch grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	Yellow leaves	–	4/351
Echinochloa pyramidalis	Antelope grass	Bungoma, Busia-K, Mbita	None	–	0/3
Eleusine indica	Crowfoot grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U	None	+*	3/86
Enteropogon macrostachyus	Bush rye	Mbita	None	+*	1/2
Eragrostis curvula	Weeping love grass	Bungoma, Busia-K, Tarime	None	–	0/8
E. superba	Maasai love grass	Bungoma, Tarime, Mbita	None	–	0/9
Heteropogon contortus	Bunch spear grass	Mbita	None	–	0/2
Hyparrhenia cymbaria	Thatch grass	Mbita	White leaves	+	1/2
H. filipendula	Fine wood grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U	None	–	0/20
H. hirta	Thatch grass	Mbita	None	–	0/1
H. pilgerana	Thatch grass	Busia-K, Tarime, Bugiri, Busia-U	None	–	0/8
H. rufa	Thatch grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	White leaves, stunting	+*	52/125
Hyparrhenia sp.	Thatch grass	Mbita	None	–	0/1
Imperata cylindrica	Spear/sword grass	Busia-K, Tarime, Bugiri, Busia-U, Mbita	None	–	0/59
Loudetia kagerensis	'Buoywee' (Luo)	Busia-K, Bugiri, Busia-U	None	–	0/6
Melinis minutiflora	Molasses grass	Mbita	None	–	0/1
Panicum antidotale	Perennial Sudan grass	Bugiri, Busia-U	None	–	0/4
P. deustum	Broadleaf Panicum	Mbita	None	–	0/1
P. maximum	Guinea grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	None	–	0/161
Pennisetum mezianum	Fountain grass	Mbita	None	–	0/1
P. polystachion	Thin Napier grass	Tarime, Bugiri, Busia-U, Mbita	None	–	0/55
P. purpureum	Napier/elephant grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	Stunting, yellow leaves	+*	77/198
P. sphacelatum	Fountain grass	Mbita	None	–	0/1
Rottboellia cochinchinensis	Itch grass	Busia-K, Tarime, Bugiri, Busia-U	None	–	0/66
Setaria incrassata	Purple pigeon Grass	Busia-K, Bugiri, Mbita	None	–	0/3
S. sphacelata	African bristle grass	Bungoma, Busia-K, Bugiri, Busia-U, Mbita	None	–	0/7
Sorghum arundinaceum	Wild sorghum	Bungoma, Busia-K, Tarime, Mbita	None	–	0/5
S. sudanensis	…	Mbita	None	–	0/1
S. versicolor	Wild sorghum	Bugiri, Busia-U, Mbita	None	+*	3/83
Sporobolus pyramidalis	Drop-seed grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	None	+*	3/77
S. (CS) consimilis	…	Mbita	None	–	0/1
Themeda triandra	Red oat grass	Mbita	None	–	0/2
Chrysopogon zizanioides	Vetiver	Mbita	None	–	0/1
Unknown	…	Bungoma, Busia-K, Bugiri, Busia-U	None	–	0/93
Total	…	…	…		270/2,185

[a] Plant names are according to those described by Muyekho et al. (2004).
[b] Presence or absence of phytoplasmas is indicated by + and –, respectively; an asterisk (*) indicates at least one symptomless sample tested positive. Not all individuals displayed symptoms listed for the species.
[c] Number positive/number tested. Includes individuals with and without symptoms. Busia-K = Busia Kenya and Busia-U = Uganda.

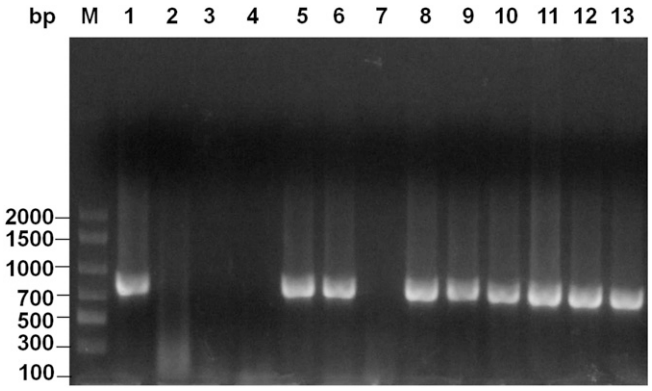

Fig. 3. Electropherogram of nested-polymerase chain reaction products amplified with NapF/NapR primers from wild grasses. Lane M, 1-kb DNA marker (GenScript, USA Inc.); lane 1, reference Napier grass stunt phytoplasma; lane 2, negative control (water); lanes 3, 4, and 7, representative wild grass plant samples negative for phytoplasma; lanes 5, 6, and 8–13, phytoplasma-infected wild grass plant samples.

GGWL, NGS, and BGWL—sharing a 99, 98.95, and 99.6% similarity, respectively, with sequences in the database.

Since GGWL was not previously reported in East Africa, a larger fragment was amplified for sequence comparison using intergenic spacer region (ISR) primers. All ISR sequences (KM924296, KM924297, and KM924298) from Mbita and Tarime districts were classified as a GGWL phytoplasma from Myanmar (GenBank accession number AB741629) sharing a 99.25% identity, confirming results from the 16S sequences. Other phytoplasmas amplified by the ISR primers were HGWL phytoplasmas (GenBank accession number KM924299). HGWL strains shared a 98.71% similarity with NGS phytoplasma (GenBank accession numbers JQ868443 and JQ868440) and a 97.35% sequence similarity with a 'Psammotettix cephalotes' flower stunt phytoplasma strain BVK isolated from Germany (GenBank accession number HQ589192). HGWL also shared a 96% sequence similarity with BGWL phytoplasma (GenBank accession number AF248961).

The resulting 16Sr DNA sequences indicated that phytoplasmas in all *C. dactylon* species collected in Busia and Bugiri districts of Uganda were indistinguishable. These phytoplasmas were nearly identical to BGWL strains of group 16SrXIV phytoplasma (accession numbers

Table 3. Diversity of phytoplasmas in grasses and relationship between symptoms and phytoplasma detection

			Accession number			
Plant host	**Host symptoms**	**Phytoplasma identified[a]**	**16S rRNA**	**ISR**	**16S rRNA group**	**Detected/tested**
Brachiaria brizantha	Asymptomatic	NGS-Bb(Tz)	KM875525	…	16SrXI	1/40
B. brizantha	Asymptomatic	BGWL-Bb(Tz)	KM875526	…	16SrXIV	2/40
B. brizantha	White leaves	GGWL-Bb(Tz)	KM875519	KM924296	…	2/40
B. brizantha	Asymptomatic	BGWL-Bb(Ug)	KM875528	…	16SrXIV	3/98
B. brizantha	Asymptomatic	RYD-Bb(Ug)	KM875529	…	16SrXI	1/98
B. brizantha	White leaves	GGWL-BrX3(Ke)	KM875533	KM924298	…	1/6
B. brizantha	Yellow leaves	BGWL-BrX5(Ke)	KM875534	…	16SrXIV	1/6
B. brizantha	Asymptomatic	RYD-Br(Ke)	KM875535	…	16SrXI	1/74
Chloris gayana	Asymptomatic	RYD-Cg(Ke)	KM879878	…	16SrXI	1/1
Enteropogon macrostachyus	Yellow leaves	RYD-Em(Ke)	KM879875	…	16SrXI	1/2
Coix laryma-jobi	Asymptomatic	RYD-Cl(Ke)	KM879877	…	16SrXI	1/1
Cynodon dactylon	White leaves, stunted growth	RYD-Cd(Tz)	KM875527	…	16SrXI	1/107
Cynodon dactylon	White leaves, stunted growth	BGWL-K1(Ke)	KM875511	…	16SrXIV	112/393
C. dactylon	White leaves, stunted growth	BGWL-K2(Ke)	KM875512	…	16SrXIV	12/132
C. dactylon	White leaves, stunted growth	BGWL-Ug1	KM875513	…	16SrXIV	13/132
C. dactylon	White leaves, stunted growth	BGWL-Ug2	KM875514	…	16SrXIV	27/124
C. dactylon	White leaves, stunted growth	BGWL-Ug3	KM875515	…	16SrXIV	21/124
C. dactylon	White leaves, stunted growth	BGWL-Tz1	KM875516	…	16SrXIV	13/107
C. dactylon	White leaves, stunted growth	BGWL-Tz2	KM875517	…	16SrXIV	20/107
C. dactylon	White leaves, stunted growth	BGWL-Tz3	KM875518	…	16SrXIV	4/107
C. dactylon	Asymptomatic	RYD-Cd(Ke)	KM875539	…	16SrXI	1/393
Digitaria scalarum	Asymptomatic	RYD-Ds(Ug)	KM875530	…	16SrXI	3/89
D. scalarum	Yellow leaves	RYD-Ds(Ke)	KM879876	…	16SrXI	1/5
Eleusine indica	Asymptomatic	RYD-Ei(Ke)	KM975504	…	16SrXI	2/47
E. indica	Asymptomatic	BGWL-Ei(Tz)	KM875524	…	16SrXIV	1/12
Hyparrhenia rufa	White leaves, stunted growth	RYD-Hr(Tz)	KM875523	…	16SrXI	8/24
H. rufa	Asymptomatic	BGWL-Hr(Tz)	KM875522	…	16SrXIV	1/24
H. rufa	White leaves	RYD-Hr(Ug)	KM875531	…	16SrXI	2/29
H. rufa	White leaves, stunted growth	RYD-Hr(Ke)	KM875538	KM924299	16SrXI	31/72
H. cymbaria	White leaves	RYD-Hc(Ke)	KM875537	…	16SrXI	1/2
Sporobolus pyramidalis	Asymptomatic	RYD-Sp(Ke)	KM875536	…	16SrXI	1/33
S. pyramidalis	Asymptomatic	RYD-Sp(Tz)	KM875521	…	16SrXI	1/13
S. pyramidalis	Asymptomatic	GGWL-Sp(Tz)	KM875520	KM924297	-	1/13
Sorghum versicolor	Asymptomatic	RYD-Sv(Ug)	KM875532	…	16SrXI	3/82
Pennisetum purpureum	Asymptomatic	NGS- Pp1(Tz)	KM924303	…	16SrXI	2/50
P. purpureum	Asymptomatic	NGS- Pp2(Tz)	KM924304	…	16SrXI	4/50
P. purpureum	Yellow leaves, stunted growth	NGS-Pp1(Ug)	KM924305	…	16SrXI	10/57
P. purpureum	Yellow leaves, stunted growth	NGS- Pp2(Ug)	KM924306	…	16SrXI	16/57
P. purpureum	Yellow leaves, stunted growth	NGS-Pp(Ke)	KM924300	…	16SrXI	16/91
P. purpureum	Yellow leaves, stunted growth	NGS-Pp2(Ke)	KM924301	…	16SrXI	12/91
P. purpureum	Yellow leaves, stunted growth	NGS-Pp3(Ke)	KM924302	…	16SrXI	8/91

[a] Names of the phytoplasmas are given according to the type of phytoplasma followed by abbreviation of the species name and country code: RYD = rice yellow dwarf, NGS = Napier grass stunt, BGWL = bermuda grass white leaf, GGWL = goose grass white leaf, Tz = Tanzania, Ke = Kenya, and Ug = Uganda.

EU409293, AJ550984, AJ550986, AJ550985, EF444485, HE599391, HE599395, AF248961, AB052871, EU234510, AB642601, AB741630, HE599392, HE599393, and KF234570) and shared a sequence similarity ranging from 99.34 to 99.6%. Phytoplasmas detected in *Sorghum versicolor*, *H. rufa*, and *D. scalarum* collected from Uganda were closely related to the NGS strains formerly reported in Kenya and Uganda (GenBank accessions numbers AY377876, EF012650, FJ862997, FJ862998, FJ862999, JQ868440, and JQ868443). Two phytoplasmas were found in *B. brizantha* sampled in Uganda. One of these had a close identity to NGS phytoplasma, with 98.95% sequence similarity, while the other phytoplasma had a 99.6% sequence similarity with BGWL phytoplasma.

Sequence analysis indicated that phytoplasmas in *P. purpureum* were identical and shared a sequence similarity of 99.21 to 100% with the 16SrXI phytoplasma strains reported previously from Uganda and Kenya (GenBank accession numbers AY377876, EF012650, FJ862997, FJ862998, FJ862999, JQ868440, and JQ868443).

Phylogenetic analysis. When compared, accessions from Tanzania and Kenya clustered into three discrete clades: the 16SrXI group, GGWL group, and 16SrXIV group. The analysis revealed that all NGS-related phytoplasmas belonged to the 16SrXI phytoplasma group but were distinct from the NGS phytoplasmas that infect Napier grass. The group was subdivided into two groups comprising phytoplasmas from Napier grass in one branch and those from the wild grasses in the other branch. All the BGWL-related phytoplasmas clustered together as one distinct group. GGWL-related phytoplasmas were also grouped together in one phylogenetic clade differing from those of the 16SrXI or 16SrXIV phytoplasma groups. Phylogenetic analysis of phytoplasma sequences from Uganda showed two distinct phytoplasma groups comprising the 16SrXI and 16SrXIV groups. Analysis of phytoplasma sequences from infected Napier grass grouped these into one full group comprising the 16SrXI phytoplasma group.

Discussion

During a survey to assess the prevalence and genetic relationship of phytoplasmas in wild grasses, more than 2,000 samples from 33 wild grass species were collected and tested. In addition to the known phytoplasma host species *C. dactylon*, *H. rufa*, and *P. purpureum* (Jones et al. 2004; Obura et al. 2010, 2011), nine grass species were found as new hosts in East Africa. Based on sequence similarities and phylogenetic analysis of 16S rDNA sequences, these phytoplasmas were divided into NGS-, GGWL-, and BGWL-related groups. The sequences from infected Napier grasses and *H. rufa* were definitively affiliated with the 16SrXI group, distinct from those associated with NGS in Ethiopia, which belongs to the 16SrIII-A group (Arocha et al. 2009; Jones et al. 2007). BGWL and HGWL phytoplasmas have been reported previously in studies from western Kenya. The studies suggested that, because of their close similarity to NGS phytoplasma, these phytoplasmas were important and posed a threat to the cultivation of Napier grass in the East African region (Obura et al. 2010, 2011). The current study has not only found evidence of a wider geographic distribution of these phytoplasmas but also has shown that these plant pathogens occur in more grass species than previously thought.

The GGWL phytoplasma has never been reported in the region in either wild or cultivated Poaceae. In this current study, GGWL phytoplasma was detected in Mbita and Tarime in two wild grasses; namely, *B. brizantha* and *S. pyramidalis*. The infected *B. brizantha* plants were white, with reduced leaf size, and stunted while the phytoplasma-infected *S. pyramidalis* plant showed no symptoms. This phytoplasma has been reported previously in Myanmar in *E. indica* (goose grass) showing similar disease symptoms like in *B. brizantha*, including white small leaves, bushy growth habit, stunting, and death of plants (Win et al. 2013b). The phytoplasma was shown to be distantly related to sugarcane white leaf (SCWL) phytoplasma, which belongs to the 16SrXI group, and other phytoplasma agents in the 16SrXIV group, but was closely related to sorghum grassy shoot phytoplasma (SGS) '*Ca.* P. oryzae'. This study showed that GGWL phytoplasma possessed other distinct features and, therefore, could not be identified into a previously described 16Sr groups (Win et al. 2013b). The current study has revealed similar findings and shown that GGWL found in symptomless and symptomatic plant species in East Africa possesses features similar to that found in Asia.

Apart from a few species, most of the wild grass hosts from which phytoplasmas were detected were indistinguishable from healthy conspecific grasses. However, phytoplasma-infected *C. dactylon* plants found in western Kenya, northern Tanzania, and eastern Uganda showed stunting with small white leaves, as observed in western Kenya (Obura et al. 2010). Likewise, HGWL-infected *H. rufa* had similar symptoms as those reported in the previous study in Kenya, displaying stunted growth with white leaves. This phytoplasma was named HGWL to depict the plant species from which it was detected (Obura et al. 2011). The findings from the current study confirm earlier reports that symptoms alone are not reliable indicators of phytoplasma presence or identity in plant hosts (Bertaccini et al. 2005; Blanche et al. 2003; Seemüller and Schneider 2007). The inability to recognize a symptomless plant harboring a phytoplasma could also result in inadvertent exposure of cultivated plants in East Africa to a potential inoculum source, as has been suggested in Australia (Blanche et al. 2003).

Besides, detecting phytoplasmas in symptomless plants (Bertaccini et al. 2005; Blanche et al. 2003; Seemüller and Schneider, 2007), the location of a phytoplasma in a plant may also vary over time (Blanche et al. 2003). For example, Blanche et al. (2003) showed variation in the occurrence of SGS phytoplasma in different parts of *Whiteochloa cymbiformis* and *Sorghum stipoideum* by searching for the phytoplasma at different times of the year. Therefore, it is possible that the location of phytoplasmas in wild grass species identified here is also variable within plants and over time. It also shows that the potential reservoir of phytoplasmas in East African grasses may be larger than these surveys suggest. The movement, distribution, and colonization of phytoplasmas in plants are theoretically systemic; however, in reality, it is not. Particularly grasses can grow quickly in a short time and negative PCR results might often be encountered when fresh leaf samples are taken because they have not yet been colonized. It might therefore be useful in future studies to examine other parts of the plant including stems, shoot apex, and roots, as have been examined in other phytoplasma-infected plants (Saracco et al. 2006; Wei et al. 2004), to indicate more reliable locations for phytoplasma detection.

Several studies have shown the role of wild grasses and weeds as alternative phytoplasma hosts and implicated them in disease epidemiology (Arocha et al. 2009; Blanche et al. 2003; Obura et al. 2010, 2011). This may be true, as many wild host plants may remain free from obvious phytoplasma disease symptoms due to coevolution with their phytoplasmas (Caudwell 1983). However, a new phytoplasma disease may emerge directly when recently introduced plant becomes a suitable feeding host for a naturally occurring vector or indirectly when a new competent vector is introduced into a habitat where both the natural and the cultivated host exist. This therefore may lead to disease outbreaks in cultivated crops, enabling the phytoplasma to exploit a new ecological niche. In such cases, genetically distinct phytoplasma strains may result if the secondary epidemiological cycle is isolated from the original system (Caudwell, 1983; Lee et al. 1998). Therefore, the current findings are not only informative but also suggest that these wild grass hosts are important and could be acting as wild sources of inocula for nearby fields planted with Napier grass, cultivated crops, and other monocots. An introduction of a competent vector into the habitat may initiate a three-way phytoplasma epidemiological system (Lee et al. 2003; Obura et al. 2009) resulting in the emergence of phytoplasma diseases with significant impacts on both wild and cultivated plants in the East African region.

While some wild grasses host only one strain of phytoplasma, others host multiple strains. For instance, two phytoplasmas were found in *C. dactylon*, *E. indica*, *H. rufa*, and *S. pyramidalis* while three of them were detected in *B. brizantha*. However, there was no indication of multiple simultaneous infections. It is likely that double or multiple infections also occur in the plants examined. However, they could not be detected by the methods employed. Additionally, NGS-related phytoplasmas were detected in 10 different

plant species, BGWL in 3 plant species, and GGWL in 2 plant species. This is possible since the specificity of the phytoplasma for the insect host is usually stricter compared to the plant host ranges (Lee et al. 1998).

The wild grasses identified here as phytoplasma hosts often grow copiously in fields bordering those planted with Napier grass. However, phytoplasmas that are closely related to BGWL and GGWL have not yet been transmitted to Napier grass. Similar findings have been reported in Australia, where SCWL was not found in sugarcane plants tested (Blanche et al. 2003) despite wild host plants that occurred nearby. Because these phytoplasmas were not detected in Napier grass collected, the infection of wild grasses with the 16SrXIV or GGWL phytoplasma may involve another, as-yet-unknown vector, suggesting their disassociation with NGS. While the vector responsible for the transmission of NGS from phytoplasma-infected Napier grass to a healthy plant was reported recently in Kenya (Obura et al. 2009), there is no record of the vectors responsible for the transmission of phytoplasmas in many wild grasses reported in this study. However, transmission is probably via sap-sucking insect vectors, mostly by leafhoppers (Cicadellidae, Hemiptera) and planthoppers (Delphacidae, Hemiptera), as with other plants (Weintraub and Beanland 2006). Therefore, further studies are needed to identify insect vectors and examine the likelihood of phytoplasma transmission to wild and cultivated monocots.

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