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Napier grass stunt disease prevalence, incidence, severity and genetic variability of the associated phytoplasma in Uganda

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ABSTRACT

The prevalence, incidence and severity of Napier grass stunt disease (NGSD) caused by phytoplasma on *Pennisetum purpureum*, the main fodder for livestock under intensive and semi-intensive management systems in Uganda were determined following a field survey carried out in 17 districts. A total of 298 Napier grass fields were visited and NGSD status visually assessed and 1192 samples collected for identification and confirmation of the phytoplasma by polymerase chain reaction (PCR) assays using universal primers P1/P6 nested with $R16F_2n/R16R_2n$ and, *tuf* primers 890/340 nested with 835 and 400. From these, 221 PCR products were sequenced and sequences aligned. Napier grass stunt disease is widely spread at an epidemic proportion, with the districts at different risk levels. The most affected districts are in central, East and North parts of the country while those in the west are least affected. Sequence alignments and Blast searches showed that the phytoplasma causing NGSD in Uganda belonged to the phytoplasma group 16SrXI, with single nucleotide sequence variants in a few districts. Therefore, there is a need for development of an area wide NGSD management strategy to contain the disease.

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1. Introduction

Napier grass (*Pennisetum purpureum*), also known as elephant grass, is the main fodder for livestock under intensive and semi-intensive management systems in Uganda (Kabirizi et al., 2007; Kawube et al., 2014). Napier grass stunt disease is, however, severely limiting Napier grass production, with its effect ranging from partial reduction in biomass yield and quality to nearly total crop loss (Alicai et al., 2004; Nielsen et al., 2007). Many smallholders have lost up to 100% of their Napier grass crop and are forced to reduce the number of animals or purchase fodder from the local market (Arocha and Jones, 2010). The disease is caused by Phytoplasma of group 16SrXI, '*Candidatus* Phytoplasma oryzae' in Uganda (Nielsen et al., 2007) and in Kenya (Jones et al., 2004) while phytoplasma belonging to group 16SrIII, Western-X-disease (particularly subgroup A) has been reported in Ethiopia (Jones et al., 2006; Arocha et al., 2009) causing unique symptoms identical to those caused by NGSD in Napier grass in Uganda and Kenya (Arocha and Jones, 2010). The symptoms include bushy appearance, yellow to purple streaking, little leaves, proliferation of tillers and shortening of internodes to the extent that clumps appear severely stunted and low biomass yield (Jones et al., 2004). However, the level of symptom expression in phytoplasma infected plants partly depends on strain virulence, strain interference, phytoplasma concentration (Marcone, 2010), abundance of insect vectors and phytoplasma infested host plants (Sharon et al., 2005). For example, Sinclair and Griffiths (2000) revealed that when periwinkle plants were co-inoculated with two strains of Ash yellows phytoplasma (*Candidatus* Phytoplasma Fraxinus – Ash yellow group), the strains greatly differed in aggressiveness, with the most aggressive strain appearing sooner and more frequently than the less aggressive strain.

The primary means of NGSD spread is through introduction of infested planting materials (cuttings or clump splits) by farmers

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and/or infected vector carrying the phytoplasma (Orodho, 2006; Koji et al., 2012). Although the vector transmitting NGSD in Uganda is not known, Obura et al. (2009) reported *Recilia banda* Kramer to be the vector of Napier stunt phytoplasma in Kenya. Indeed Koji et al. (2012) found this vector abundant in Napier grass fields in western Kenya. Given the close proximity of the two countries, it is likely that the vector is also abundant in Uganda, transmitting the disease.

Development and deployment of management tactics against any disease in a geographical area is guided by quantitative information on the existing levels of disease risk, definitive identification of the pathogen and clear knowledge of factors that correlate strongly with disease/pathogen risk within a defined host population (Thébaud et al., 2009). At present, there is no reliable information on the phytoplasma strains causing NGSD, its distribution and that of NGSD within and among Napier grass fields in Uganda. When established, such information can indicate large scale risk factors associated with high NGSD risk in an area and guide the development of management options against the disease. This study, therefore, determined the prevalence, incidence, and severity of NGSD and the strains of phytoplasma causing NGSD in Uganda.

2. Materials and methods

2.1. Incidence and distribution of Napier grass stunt disease in Uganda

Two hundred ninety eight farmer' fields of Napier grass in 17 districts of Uganda (Fig. 2) were surveyed during June and July, 2009 to assess NGSD status. The districts were selected because they are known for practicing intensive and semi-intensive livestock keeping. In each district, 10 to 20 Napier grass fields were arbitrary selected in 3–5 parishes and NGSD presence determined by visually inspecting 4 randomly selected plants along the diagonal of each field. Disease severity was assessed on a scale of 1–4, where 1 = no disease; 2 = plant diseased and slightly stunted; 3 = diseased and moderately stunted and 4 = diseased and severely stunted (Alicai et al., 2004). For each field, the position coordinates were also recorded using GPS and used to generate NGSD risk map of Uganda using ArcGIS software (ESRI, Redlands, CA). For each Napier grass inspected, a sample (a shoot) was also picked, collecting a total of 1192 samples for molecular detection of the phytoplasma in the laboratory.

2.2. Molecular detection of phytoplasma

In the laboratory at the National Crops Resources Research Institute, Namulonge, 385 subsamples were randomly selected from the collected samples representing all the districts under study. Total plant DNA was extracted from about 1.5 g of each, following the CTAB method (Doyle and Doyle, 1990) with slight modification as follows; Each Napier grass leaf sample was ground in into a fine powder in liquid nitrogen and immersed into 2% CTAB extraction buffer (20 mM EDTA, 0.1 M Tris–HCl pH 8.0, 1.4 M NaCl, 2% CTAB, plus 1% β-mercaptoethanol) in a 1.5 mL tube. The mixture was then vortexed for 10 S and incubated at 60 °C for 30 min. Chloroform-isoamylalcohol (24:1) was added to the mixture, vortexed for 10 S and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube and cold isopropanol (−20 °C) added. This was followed by gently mixing the liquid by inversion and thereafter centrifuged at 13,000 rpm for 10 min. The DNA pellet adhered to the bottom of the tube. The liquid phase was decanted and DNA washed twice in 70% ethanol by centrifuging it at 13,000 rpm for 3 min and decanting the ethanol. The pellets were dried at room temperature by inverting the tube for 12 h. The extracted DNA was diluted in 100 μl of double distilled water from which template DNA was taken for subsequent PCR reactions.

Polymerase chain reaction was carried out in 25 μL of master mix containing 1 μL of each primer, 0.2 μL of dNTPs (10 nm), 2.5 μL of polymerase buffer promega), 1.5 μL $MgCl_2$ (25 nm), 17.6 μL of sterile distilled water, 0.2 μL of *Taq* polymerase (Promega) and 2 μL of (25 ng/μL) template DNA. The following primers and conditions were used: For amplification of a region of the *tuf* gene, primer cocktails of *tuf*890/*tuf*340 were used in the first round. The products were diluted to 1:20 ratio with sterile distilled water and nested with primer cocktail *tuf* 835/*tuf*400 (Makarova et al., 2012). Each primer cocktail consisted of several variants of the same primer mixed in equal proportion (Table 2) at a concentration of 5 pmol. For both reactions, the following conditions were applied; 94 °C (2min), followed by 35 cycles of 94 °C (30 s), 53 °C (1 min) 72 °C (1.5 min), followed by 72 °C (15 min). To amplify the 16Sr gene, primers p1/p6 were used with the following conditions: 94 °C (3 min), followed by 35 cycles of 94 °C (30 S), 53 °C (1 min) 72 °C (1.5 min), followed by 72 °C (10 min). The PCR products were diluted to 1:20 ratio and nested with primers $R16F_2n/R16R_2n$ using the following conditions: 94 °C (2min), followed by 35 cycles of 94 °C (1 min), 50 °C (2 min) 72 °C (3 min), followed by 72 °C (10 min) (Table 1). The PCR products were separated by electrophoresis on 1% agarose gel stained with ethidium bromide and run for 60 min at 13Amps and 80V of electricity. The gel was visualized on a UV transilluminator. Two hundred forty six samples generated bands of size 1200 bp for p1p6/$R16F_2n/R16R_2n$ and about 400 bp for *tuf* primers *tuf* 835/*tuf* 400 and were thus regarded as positive with Napier stunt phytoplasma. A total of 221 positive PCR products (for the two regions) were sequenced for both strands at Macrogen Inc. (Seoul, Korea) using primers $R16F_2n/R16R_2n$ and, M13F and T7 for the 16Sr and *tuf* regions, respectively.

2.3. Data analysis

2.3.1. Field data analysis

Disease prevalence in each district was calculated as the percentage of fields with NGSD out of the total number of fields inspected. Disease incidence (based on visual symptoms) was calculated as the percentage of NGSD symptomatic plants out of the total number of sampled plants in each field, while for molecular data incidence was calculated as samples tested positive for phytoplasma divided by the total number of samples tested in each district. All data collected during the survey were analysed to generate variance, mean components of disease severity, incidence

Table 1
Primers used in amplification of Napier grass stunt disease.

Primer ID	Oligonucleotide sequence	Source
tuf 340a	GCTCCTGAAGAAARAGAACGTGG	Makarova et al., 2012
tuf 340b	ACTAAAGAAGAAAAAGAACGTGG	
tuf 890a	ACTTGDCCTCTTTCKACTCTACCAGT	
tuf 890b	ATTTGTCCTCTTTCWACACGTCCTGT	
tuf 890c	ACCATTCCTCTTTCAACACGTCCAGT	
tuf 835a	AACATCTTCWACHGGCATTAAGAAAGG	
tuf 835b	AACACCTTCAATAGGCATTAAAAAWGG	
tuf 835c	AACATCTTCTATAGGTAATAAAAAAGG	
tuf 400a	GAAACAGAAAAACGTCAYTATGCTCA	
tuf 400b	GAAACTTCTAAAAGACATTACGCTCA	
tuf 400c	GAAACATCAAAAAGACAYTATGCTCA	
tuf 400d	GAAACAGAAAAAAGACAYTATGCTCA	
tuf 400e	CAAACAGCTAAAAGACATTATYCTCA	
$R16F_2n$	GAAACGACTGCTAAGACTGG	Gundersen and Lee, 1996
$R16R_2n$	TGACGGGCGGTGTGTACAAACCCCG	
P1	AAGAGTTTGATCCTGGCTCAGGATT	Deng and Hiruki, 1991
P6	CGGTAGG GATCACTTGTTACGACTTA	

Fig. 1. Napier grass infected with NGSD showing leaf yellowing, purple streaking, proliferation of tillers and stunting (A and B) and a Napier grass presumed to be healthy (C).

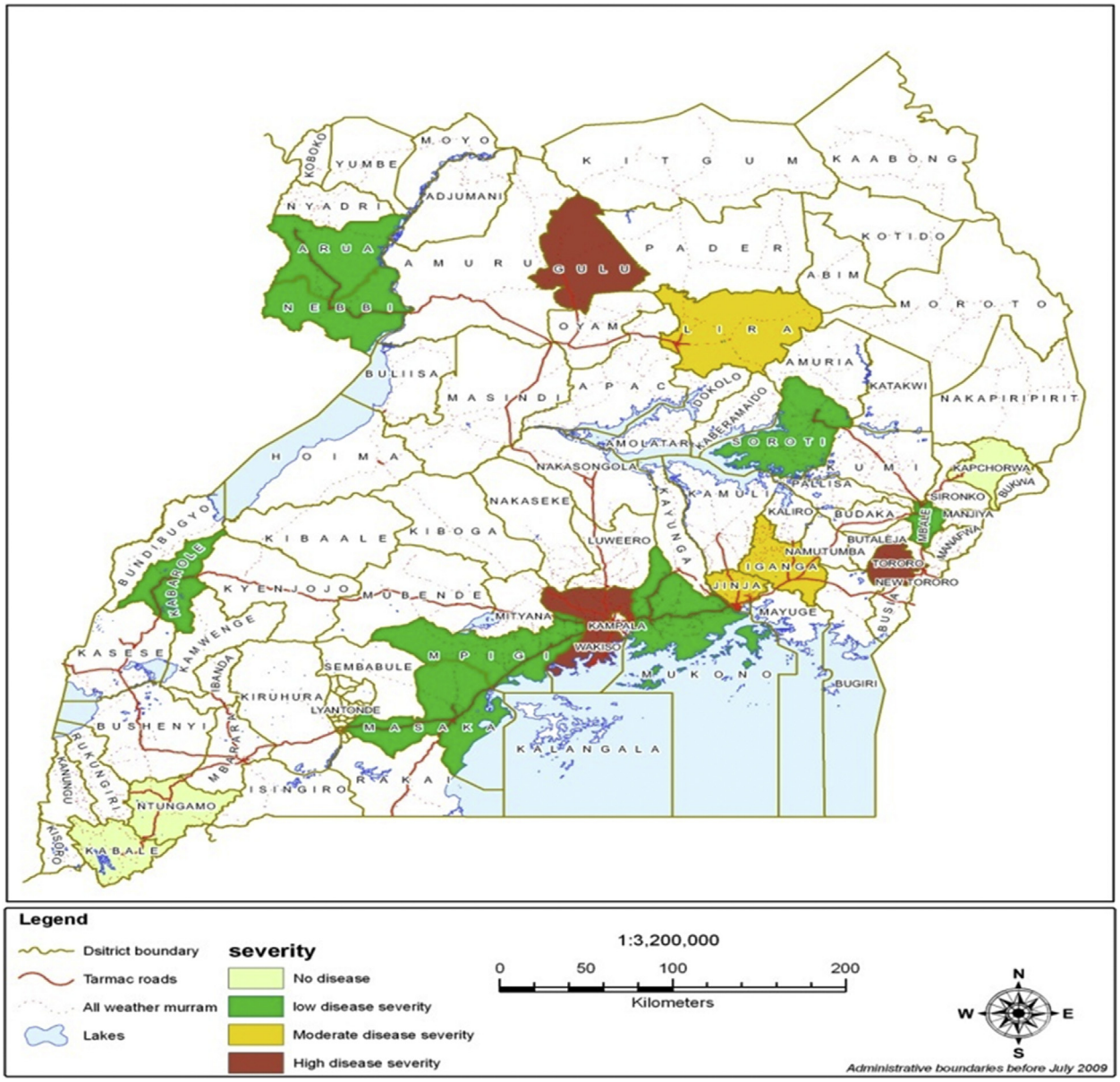

Fig. 2. Map of Uganda showing disease status of the sampled districts based on visual assessment. No NGSD was observed in the districts of Kabale and Ntungamo yet PCR amplification revealed samples infected with phytoplasma that causes NGSD.

Table 2
Prevalence, incidence of NGSD and altitude of areas where Napier grass samples were collected in Uganda.

Districts	Number of fields sampled (n)	NGSD prevalence (%)	NGSD incidence (%)	Altitude range (M.a.s.l)
Arua	17	18.0(3)	75.0	1040–1339
Nebbi	11	18.0(2)	50.0	1367–1451
Kapchorwa	20	0.0(0)	0.0	1795–1920
Ntungamo	12	0.0(0)	0.0	1222–1523
Kabale	20	0.0(0)	0.0	1522–1913
Masaka	20	25.0(5)	55.0	1200–1266
Mpigi	20	25.0(5)	75.0	1174–1257
Wakiso	20	100.0(20)	82.5	1134–1207
Mukono	20	40.0(8)	59.4	1118–1177
Jinja	20	75.0(13)	66.7	1152–1219
Iganga	20	65.0(13)	71.2	1076–1148
Mbale	20	55.0(11)	52.3	1120–1191
Soroti	14	36.0(5)	40.0	1042–1123
Lira	18	72.0(13)	53.9	1067–1095
Gulu	15	100(15)	73.3	1092–1127
Kabarole	20	15.0(3)	25.0	1482–1567
Tororo	11	100.0(11)	69.4	1160–1191
Cv (%)			42.0	
$LSD_{(0.05)}$			49.6	
F-test P = 0.05			Significant	

Figures in brackets indicate number of fields in which NGSD was observed.

and prevalence using the Generalized Linear Mixed model in Genstat12 (edition) statistical software (VSN International, UK). Significant mean values were separated using the Least Significant Difference (LSD) at 5%.

2.3.2. Sequence data analysis

The sequences were assembled using the CLC main workbench 6.8.1 software. The resulting consensus was subjected to BLAST at NCBI and closely related sequences were retrieved. These were aligned together with all the consensus sequences using progressive alignment algorithm (Feng and Doolittle, 1987) embedded in CLC workbench package with the following defaults: gap open cost 10, gap extension cost 1, end gap cost as any other.

3. Results

3.1. Napier grass stunt disease prevalence, incidence and severity

Napier grass stunt disease symptoms were observed either on the whole Napier grass stool or only on a few shoots on the stool.

Table 3
Frequency (%) of NGSD infestation in farmer's fields across districts of Uganda.

Districts	1	2	3	4
Arua	82	5	8	5
Gulu	7	46	47	0
Iganga	49	40	7	4
Jinja	45	47	7	1
Kabalore	96	0	3	1
Lira	56	15	25	4
Masaka	86	11	3	0
Mbale	63	25	6	6
Mpigi	80	5	6	9
Mukono	75	11	8	6
Nebbi	89	0	11	0
Soroti	78	14	0	8
Tororo	52	19	22	7
Wakiso	13	33	28	26
Kapchorwa	100	0	0	0
Ntungamo	100	0	0	0
Kabale	100	0	0	0

Rating according to Alicai et al. (2004). Numerical scale 1 – plants with no disease; 2 – plants diseased and slightly stunted; 3 – plants diseased and moderately stunted and 4 – plants diseased and severely stunted.

The symptoms included bushy appearance, yellow to purple streaking, reduced leaf growth, proliferation of tillers and shortening of internodes (Fig. 1). Napier grass stunt disease symptomatic plants were observed in 14 of the 17 districts surveyed with significantly varied incidence ($P \leq 0.05$) (Fig. 2 and Table 2). The average incidence of the NGSD symptomatic Napier grass plants was 49.9% across the 17 districts; significantly high incidence of NGSD (exceeding 65%) was recorded in Wakiso, Arua, Mpigi, Gulu, Jinja, Tororo and Iganga districts. No NGSD symptomatic plants were observed in the sampled Napier grass fields within the districts of Ntungamo, Kabale and Kapchorwa. Average NGSD prevalence was 43.8% and all Napier grass fields inspected in the districts of Wakiso, Tororo and Gulu had diseased Napier grass plants. The prevalence of NGSD in Iganga, Jinja, Lira and Mbale districts was more than 50% but less than 100%, while NGSD symptoms were observed in less than 50% of the sampled fields in Arua, Kabarole, Mukono, Nebbi and Soroti districts (Table 2).

Generally, more than 80% of Napier grass plants inspected showed no or slight disease, and subsequently were assigned disease severity scores 1 and 2, respectively. Among districts, Wakiso, Gulu, Lira and Tororo had more plants with moderate to severe disease (scores 3 and 4), while Masaka, Kabarole, Jinja and Soroti had fewer plants with moderate to severe disease. Napier grass fields in the districts of Kapchorwa, Ntungamo and Kabale did not show any NGSD symptoms (Table 3).

3.2. Molecular detection of phytoplasma

Polymerase chain reaction analysis of Napier grass samples based on the 16Sr region (Fig. 3) and the *tuf* region (Fig. 4) generated bands of the expected size on 1% agarose gel for positive samples. On this basis, phytoplasma that causes NGSD was detected in 16 of the 17 districts of Uganda. The percentage of positive samples identified in the districts using molecular means was relatively similar to what was detected by visual assessment in the districts where the disease was detected (Table 4).

The sequences generated by the 16Sr gene and subjected to BLAST analysis showed 100% identity to NGSD and NGSBS (GenBank Accession No. EF012649.1 and JQ868440.1, respectively). However, the *tuf* gene sequence aligned with GenBank NGSBS (accession number JQ824249.1) and NGS (accession number

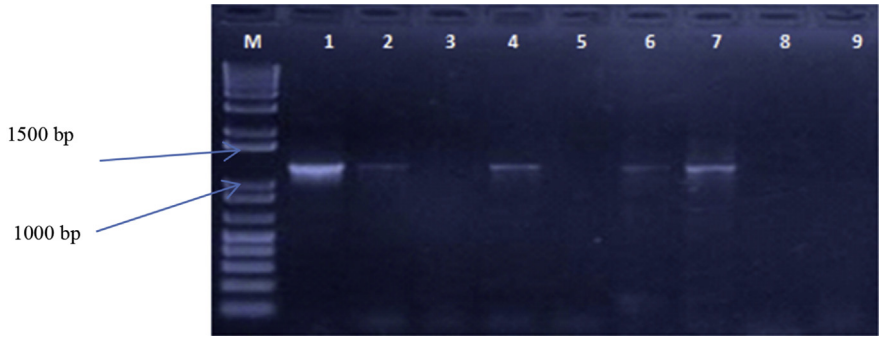

Fig. 3. PCR products of Napier grass phytoplasma obtained using p1/p6 primers nested with $R16F_2n$ and $R16R_2n$ (about 1200 bp). Lane M is 1 kb ladder; Lane 1, 2, 4, 6 are positive while 3 and 5 are negative samples; lane 7 is a positive control, lane 8 and 9 are negative control and water, respectively.

JQ824288.1) sequences revealed variation in Napier grass stunt phytoplasma by a single nucleotide. At one position, a few samples from the districts of Iganga, Jinja and Masaka had phytoplasma sequences with nucleotide A (similar to the Genbank accessions) instead of G. Phytoplasmas whose sequence had nucleotide G were widely distributed in all districts in which phytoplasma causing NGSD was detected (Fig. 5).

4. Discussion

The current major challenge to Napier grass production in Uganda is NGSD caused by Napier grass stunt phytoplasma. This study provides the first quantitative report on the incidence, severity and prevalence of NGSD and variation in Napier stunt phytoplasma in Uganda. This study indicates that NGSD and its causative pathogen –phytoplasma belonging to 16SrXI group are widespread, with districts at different risk levels. Thus, indicating the potential impact of NGSD on the livestock industry in Uganda. The most affected districts are in the central, east and northern part of Uganda, indicating existence of area-wide risk factors favouring spread of the disease. These could be abiotic (climatic, edaphic etc.) or biotic (high density of vectors, multitudes of alternative hosts such as *Hyparrhenia rufa* and *Cynodon dactylon* etc.). Thus, there is a need for an area-wide NGSD management strategy for areas at higher NGSD risk e.g growers in areas at higher risk should apply strategies that reduce NGSD build up in the fields such as controlling the insect vectors; timely weeding; fertilizer application and roguing infected plants, while those in areas at lesser risk should apply strategies such as quarantine and planting disease free Napier grass stem cuttings/or root splits to prevent spread of NGSD to other areas.

The higher NGSD prevalence, incidences and severities in the districts of Tororo, Iganga, Jinja and Mbale could be attributed to its close proximity to western Kenya where the disease was first reported (Jones et al. 2004). Indeed Asudi et al. (2015) reported high incidence of NGSD in two districts (Busia and Bugiri) in eastern Uganda, which are neighbouring these areas. The highest disease occurrence in Wakiso district could be due to the location in the district of two national agricultural research institutes which have a long history of research on Napier grass and the highest number of farmers practicing intensive livestock husbandry. Therefore, the disease might have been indirectly introduced to farmer fields through infested planting materials from elsewhere. Similarly, the high disease occurrence in Gulu and Lira could be attributed to transportation of Napier grass cuttings to the districts, most of which are obtained from research institutes in Wakiso districts. This represents the capacity of NGSD to cause epidemics in the country (Lee and Davis, 1992). Currently, the genetic variability of Napier grass in Uganda is not well studied and the two known Napier grass clones grown by farmers are susceptible to NGSD (Kawube et al., 2014). The narrow genetic base of Napier grass and the continuous growing of susceptible clones may be responsible for increased disease risk in the country. Farrell et al. (2002) attributed the devastating outbreaks of eyespot (*Helminthosporium* sp) in the Caribbean and white mould (*Beniowskia sphaeroidea*) in Kenya on Napier grass to lack of genetic variability. Although the reasons for less disease risk in western Uganda is not known, we presuppose that it is due to limited movement of Napier grass planting materials among farmers since a vast number of them practice extensive farming. Although the vector species

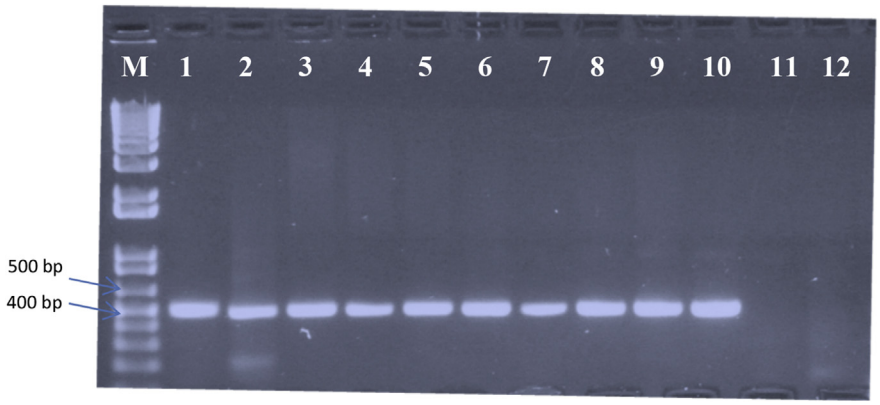

Fig. 4. PCR products of Napier grass phytoplasma obtained using *tuf* primers 340/890 nested with 400/835 (440 bp). Lane M is 1 kb ladder; Lane 1–9 are positive Napier grass samples; lane 10 is a positive control, lane 11 is water and lane 12 is a negative control.

Table 4
Percentage of Napier grass samples positive for phytoplasma as determined by nested PCR.

District	Number of samples tested	Number of samples tested positive	Percentage of positive samples
Arua	21	14	66.7
Nebbi	19	9	47.4
Kapchorwa	12	0	0.0
Ntungamo	18	7	38.9
Kabale	19	7	36.8
Masaka	25	16	64.0
Mpigi	27	17	62.9
Wakiso	44	36	81.8
Mukono	22	12	54.6
Jinja	28	22	78.6
Iganga	23	19	82.6
Mbale	17	11	64.7
Soroti	19	10	52.6
Lira	24	19	79.2
Gulu	25	21	84.0
Kabarole	17	7	41.2
Tororo	25	19	76.0

responsible for transmission of Napier grass stunt phytoplasma in Uganda are not yet known, *Recilia banda* (*Aauchenorryncha: Cicadellidae*) has been reported by Obura et al. (2009) to transmit NGSD and Koji et al. (2012) found it in high abundance in western Kenya. Therefore, there is a high likelihood that the vector exists in Uganda too, playing a major role in the spread of the disease within and between fields.

Identifying and characterizing the pathogen is often the first step in describing the epidemiology of a phytoplasma-associated disease, particularly as specific species or strains may have a specific biology that is different from other similar or closely related phytoplasmas (Constable, 2010). According to Davis and Sinclair (1998) disease diagnosis based on visual symptoms is inappropriate as it may lead to erroneous diagnosis and as such misdirect efforts at disease management. Besides, symptom development among others depends on the virulence of the infecting phytoplasma strain within a given taxon (Marcone, 2010) which is difficult to determine using visual assessment. This could explain the failure to detect NGSD in Kabale and Ntungamo district. Similar observations were previously made for yellow leaf syndrome of sugarcane in South Africa (Cronjé et al., 1998) and in Cuba for sugarcane and weeds (Arocha et al., 2005). The absence of disease and the phytoplasma in Kapchorwa district is probably due to minimal movement of planting materials from the neighbouring districts infected with NGSD, thus disease development in this district could be just a matter of time.

Molecular-based analyses introduced during the last two decades have proven to be more accurate and reliable for phytoplasma identification (Lee et al., 2000) than visual inspection. The 16Sr RNA gene is widely used in taxonomic and evolutionary studies and an arbitrary threshold of 2.5% dissimilarity was applied as a guideline for electing a new species (IRPCM, 2004). On this

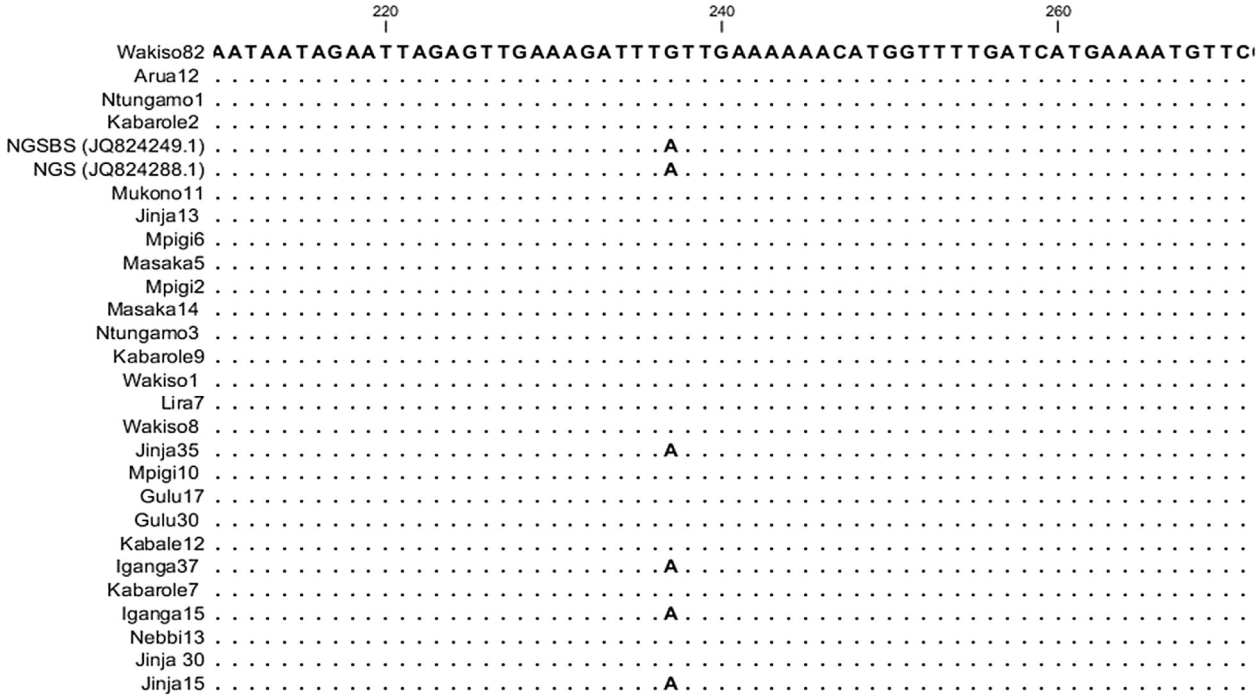

Fig. 5. Alignment of a section of a partial *tuf* sequence between annealing positions of *tuf* primer 835 and 400 for the Napier grass stunt phytoplasma used in this study along with NGSBS (GenBank accession no. JQ824249.1) and NGS (GenBank accession no. JQ824288.1). Sequences were aligned using CLC main workbench. Dots represent identical concensus nucleotides while A represents a nucleotide similar to that of sequences retrieved from NCBI.

basis Nielsen et al. (2007) identified phytoplasma of group 16SrXI, '*Ca.* Phytoplasma oryzae) as the causal agent of NGSD in Uganda basing on Napier grass samples collected from Masaka district in Uganda. Similar findings were obtained in this study based on samples collected from 16 districts. However, there are deficiencies in the phylogenetic system for phytoplasmas based solely on a single, highly conserved, non-coding gene such as the 16S rRNA, especially for defining subgroups within a species (Streten and Gibb, 2005). The *tuf* gene analysis revealed variation in the sequence of phytoplasma causing NGSD in Uganda by one nucleotide from the already known sequences. The variant is widely spread in all the districts infested with NGSD, with only districts of Iganga, Jinja and Masaka having a low incidence of phytoplasma with already known sequence. But assigning the variant to subgroup is still subject to scientific inquest. According to Davis and Sinclair (1998), this ought to await incorporation of other genomic and phenotypic data that could indicate distinct lineages. Related studies by Arnaud et al. (2007) reported existence of three cluster strains of *Flavescence doree*; with cluster strain 1 common in south western France, cluster strain 2 common in France and Italy and, cluster strain 3 found only in Italy.

5. Conclusion

There is NGSD epidemic in Uganda with the districts at different risk levels. The most affected districts are in central, east and north parts of the country. The disease is caused by a phytoplasma belonging to the 16SrXI group with a variant (based on the *tuf* gene) more widely distributed in the country than the known type. Therefore, as Napier grass production in Uganda is increasing, there is a need for development of an area wide NGSD management strategy to contain the disease. In the interim, efforts should be geared towards increased awareness of NGSD and deployment of phytosanitary measures to control further spread of the disease among communities where NGSD has not been reported.

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