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Molecular Diversity of a Seemingly Altitude Restricted *Ustilago kamerunensis* Isolates in Kenya: A Pathogen of Napier Grass

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Abstract

The Central region of Kenya which is the second largest market oriented dairy zone, faces a threat in milk production. The challenge is a disease known as the napier head smut caused by *Ustilago kamerunensis*. This fungal microorganism is a facultative pathogen which has been reported to cause yield losses in napier grass (*Pennisetum purpureum*) ranging from 25% to 46% across the affected areas. Additionally, there are reports of the continual spread of the disease into neighbouring county of Nakuru in Rift-Valley region which is the leading milk producing zone in the country. This scenario of spread is worrying combined with observation of variations in damage levels of napier grass clones across the five counties of Central Kenya. These observations led to the hypothesis that possible differences might be existing among the *Ustilago kamerunensis* variants in Kenya. Further, the differences in biomass yield losses that are within a certain percentage range mentioned-above, seemed to support the existence of possible differences. Therefore, to inform effective integrated management strategies of the pathogen in case it's co-evolving, this study sought to determine the molecular differences of *Ustilago kamerunensis* isolates in affected counties using ITS 1 and 2 regions which are spanned by 5.8S ribosomal RNA gene. The *Ustilago kamerunensis* propagules were systematically collected from affected counties' hot spot areas for sequencing and phylogenetic analysis. The study revealed the most affected areas to be within the mean altitude level of 1988.17 ± 71.97 metres above sea level. Further, differences in the growth *in vitro* and molecular characteristics of the seemingly

altitude restricted isolates were observed. The Kiambu, Nyandarau and Nakuru counties isolates clustered together, whereas those of Murang'a, Nyeri and Kirinyaga formed another clade. The sequences of sixteen *Ustilago kamerunensis* isolates were deposited in GenBank with accession numbers ranging from MG722754 to MG722769. The results suggest the existence of possible genetic divergence of the isolates which might be reflected in their pathogenic potential too. Effective integration of management strategies is vital towards slowing the phenomenon for an optimal mitigation of the disease in Kenya.

Keywords

Ustilago kamerunensis, Diversity, Altitude, Napier Grass, Milk

1. Background

The Central region of Kenya is a high potential market oriented dairy zone second to Rift-valley [1] [2]. The region relies heavily in napier grass (*Pennisetum purpureum*) as a source of feed of the livestock with over 70% of the smallholder dairy farmers growing the crop [3] [4] [5]. However, in recent years the dairy industry is threatened by emergence of a disease called napier head smut caused by a facultative pathogen called *Ustilago kamerunensis* [6]. The pathogen has been reported to cause a varying degree of damage on affected crop across the counties of the region ranging between 25% - 46% yield losses [1]-[7]. Further, the varying observations in damage levels have led to the presumptions revolving around the likely existence of strains of the pathogen which are more virulent than others or the country's cultivars being more susceptible to the disease [8] [9]. Moreover, of concern has been its continual spread to other parts of Kenya like the Rift-valley which is the leading milk producing region [10]. Among the major factors contributing to the spread is the production of spores called ustilospores from smutted heads of susceptible cultivars and germplasm cuttings exchange between farmers from affected to unaffected areas as seed is unaware that the systemic intercellular pathogen is being carried along within the susceptible crop's tissues [4] [11] [12]. Therefore, to inform effective integrated management strategies of the pathogen in case it's co-evolving in this high potential milk producing regions, this study sought to determine the molecular differences of *Ustilago kamerunensis* isolates in the affected counties using ITS 1 and 2 regions which are spanned by 5.8S ribosomal RNA gene.

2. Napier Grass and the Napier Head Smut Disease

2.1. Why Napier Grass (*Pennisetum purpureum*)?

Pennisetum purpureum Schum., commonly known as napier grass is a fodder crop of great economic value in the tropical regions [13] [14]. The forage crop is

highly adopted in Eastern and Central Africa due to its high biomass outputs, rapid regrowth after harvesting and ease of chewing by livestock in their leafy stage [15] [16] [17] [18] [19]. Also, in Kenya the crop is a major feed source of small scale dairy farmers either through fresh harvest or as silage forms [7] [14]-[20]. The small scale dairy farmers supply 80% of the total marketed milk nationally [1]-[7]. As a result, its cultivation has been on the rise in a positive correlation with the dairy industry's growth [7] [8] [9] [10] [11].

2.2. The Napier Head Smut

Napier head smut is caused by *Ustilago kamerunensis* a smut fungus that belongs to the *Ustilago* genus. The fungus belongs to division eumycota and sub-division basidiomycotina which are characterized by their bi-nucleate spores and formation of a dikaryon from vegetative part's fusion [21] [22]. The smut fungus sub-division is second to rusts' (pucciniales formely uredinales) in the division in terms of species numbers that have a high economic implication [23]. The fungus belongs to the class basidiomycetes, sub-class heterobasidiomycetidae which is characterized with numerous identified orders that give rise to over 77 genera under smut and bunts [24] [25] [26] [27] [28].

2.2.1. Etiology of the Napier Head Smut Disease

Ustilago kamerunensis the causative agent of napier head smut grows within the plant's cells and slowly spreads systemically to the entire plant's tissues. Its hyphae that are branched with internal partitions (septate) produce lobed and curved haustoria that form the feeding structures of this parasite in the host plant or it can feed directly through the cell walls. Its ustilospores are sub-globose with an estimated 7 μm diameter. At reproduction the spikelets confine the sori with the ustilospores becoming a black loosely attached mass for easy dissemination [3]-[29]. Because of this the reproductive investment by this systemic pathogen using the host's resources is quite significant that it reduces the plant's biomass extensively [30]. This is compounded by the perennial life cycle of the pathogen where it produces ustilospores continuously in huge amounts to the soil [3]. Hence, once a field is infected then for sure one has to ensure the likely management strategy is thorough if the disease problem is to be ameliorated.

2.2.2. Epiphytology of the Napier Head Smut Disease

Epiphytotics of napier head smut can be attributed to certain abiotic conditions like; temperature range of between 5˚C and 35˚C with an optimum witnessed around 20˚C highly favouring the establishment of this pathogen. Moreover, high relative humidity ranging between 65% - 90% enhances the disease's initiation on susceptible host. This is after successful *Ustilago kamerunensis* spread from a sick crop to health susceptible one that is primarily facilitated by wind transfer of ustilospores from smutted inflorescence to new unaffected areas compounded by ustilospores inoculum on the field soil in natural infections

scenario [3]. Secondary transmission of the pathogen is through; animal carrying stuck ustilospores on them, animal's waste fed on the smutted crop, clothes of passersby and planting of diseased canes carrying the pathogen within their tissues [3] [4] [11] [12]. The most susceptible stage of the crop is during the development stage of the buds into shoots (shoot infection) of a respective cane or when the buds are pushing through the soil a factor explaining why the disease is so severe in the regrowth of a second crop after the first harvest, due to the many buds that provide extensive shoots to infect and the damaged stem tissues which also provide entry points of the pathogen [3].

2.2.3. Napier Head Smut Disease Symptoms

The disease firstly manifests itself in susceptible hosts through induced premature flowering covered in a black mass of ustilospores commonly referred to as the smut. This occurs even in plants that are below 1.5 m height which is not usually the case in health plants that usually flower in heights above 1.5 to 8 metres depending on the variety of the grass, with others even taking so long to do so due to a very long vegetative phase [3]-[15]. This visual sign is later compounded by other severe symptoms up on first harvest and regrowth influenced largely by the levels of susceptibility of the grass type including; slow regrowth after cutting, withering and chlorosis setting in with gradual browning towards drying and death of the entire stool of the crop within the subsequent 2 - 3 cuttings in severe cases [11] [12]. Besides the above primary signs other secondary characteristics of the disease like; induced dwarfing (stems are thinner and shorter than normal less than 1.5 m in height) has been observed in serious cases, characterized by short internodes with distorted leaves in shape that are reduced in number and size on stools, with an increased tillering scenario [3] [4]-[12].

2.2.4. Napier Head Smut Disease's Distribution and Research Status

Research on ways of mitigating napier head smut is limited a phenomenon that can be attributed to ignoring of this vital crop by virtue of it being a feed source for livestock [8]. In Kenya the entry route of the disease to the country is mapped from West Africa, through Uganda (1930), Rwanda (1963), Tanzania (1975), and eventual establishment in the country in the 1990s where it was first reported in press affecting Central's Lari division in Kiambu district by Kung'u and Waller [3] [9]-[31]. Since then its distribution within the several divisions of the region has been very notable and logarithmic [3]; [4]-[9]. The spread is compounded by some worrying reports of its severe occurrence in some new parts of the country like; in Rift-valley at Molo and Londiani and the lower Eastern region at Meru north and south [10]-[32]. Furthermore, reports of a possible co-evolutionary scenario due to the resistance pressure on the head smut pathogen leading to emergence of possible new variants has provoked the need for characterization of the head smut pathogen in Central Kenya [29]. Thus, how wide the selected tolerant accessions can be cultivated and adopted on different

agro-ecological zones successfully in the country cannot be guaranteed with certainty due to a possible breakdown of resistance if diverse isolates exist [1]; [11]-[33].

3. Materials and Methods

The *Ustilago kamerunensis* isolates were collected in Central-Kenya hot spot areas with the guidance of KALRO-Muguga south experts. The laboratory assays were conducted at the International Centre for Insect Physiology and Ecology-Mbita, located on latitudes (0˚25'S & 0˚30'S) and longitudes (34˚10'E & 34˚15'E) at an altitude of 1200 metres above sea level.

3.1. Collection, Culturing *In Vitro* and Sequencing of Head Smuts' Isolates Genomic DNA

A modified purposive sampling strategy was carried out across the six counties' napier head smut hot spots *viz*.; Kirinyaga, Nyandarua, Nyeri, Kiambu, Murang'a and Nakuru as reported by [1]-[10]. Thus, in a respective field over 22 kilometres apart within each county a smutted napier bush was selected at the mid-point of a x-shaped transect stretching between opposite ends of the field within a range of 0.5 to 5 metres radius depending on the size of the fields. The isolates of napier head smut ustilospores were then collected from the individual bush to limit collection of mixed isotypes in case of multiple isotypes infection of an individual field for molecular characterization using a modified approach [33]. The isolates on collection were given names starting with the three initials of the county and a number which signified the percentage of smutted napier grass stools within a particular sampled farm. For instance (001 or 1), (002 or 2), (003 or 3), (004 or 4), (005 or 5), (006 or 6), (007 or 7), (008 or 8), (009 or 9) and (010 or 10) mean't that the isolate was collected from a field whose smutted napier grass stools incidence was ≥90% -100%, ≥80% - 89%, ≥70% - 79%, ≥60% - 69%, ≥50% - 59%, ≥40% - 49%, ≥30% - 39%, ≥20% - 29%, ≥10% - 19% and ≥0% - 9% respectively. Three different isolates were collected from each county and assigned the appropriate number depending on the criteria above. The geographic position coordinates and altitude of the area was recorded using etrex garmin geographic positioning system tool to aid mapping of the isolates using ArcMap application of the ArcGIS. The spores were collected by cutting the smutted heads using a pair of scissors and putting them in pollination bags which were then shaken manually to remove the spores from the inflorescence [29]. These materials were placed in pollination bags and taken to the laboratory where they were stored at a cool dry place at 25˚C awaiting culture *in vitro*.

3.2. Culturing of the Ustilospores from Napier Head Smut Isotypes *In Vitro* towards Genomic DNA Extraction

The respective head smut isolates' ustilospores were cultured on 10 ml petri dishes containing sterilized oxoid malt extract agar at 121˚C for 15 minutes [34].

This media was treated with 10 ml lactophenol per litre during preparation to inhibit bacterial growth. Then a 10 µl volume pre-standardized pathogen spore inoculum concentration of 5×10^6 spores $m \cdot l^{-1}$ was spot inoculated at the centre of each plate under a lamina air flow chamber [35] [36]. The inoculations for each isolate was replicated 10 times in a completely randomized design. The inoculated plates were then incubated at 25˚C upon sealing them using a parafilm in a dark area. After, 4 days of culture which is the minimum recommended culture period for fungal microorganisms at 25˚C [37]. The colony growth average diameter was determined at this point before it fully colonized the petri plate, to aid assess the vigor in growth *in vitro* of the isolates. This *in vitro* culture of the isolates was repeated to validate the outcome. Then, they were sub-cultured to obtain pure cultures of the head smut isolates that exhibited; top white floccose and reverse pale cream colonies. The colonies were then used in the extraction of their genomic DNA for sequencing and subsequent phylogenetic analysis.

3.3. Genomic DNA Extraction and Amplification

Total DNA was extracted from the respective *Ustilago kamerunensis* isolates' colonies using a modified Bioline® Isolate II Genomic DNA extraction kit [38]. Towards the extraction lysis buffer G3, wash buffer GW2 and proteinase K was prepared as per the kit manual directions. Then 75 mg of the respective fungal colonies was thoroughly ground using a different mortar and pestle for each isolate. Due to the unique fungal structure 0.5 ml extraction buffer was added to enhance extraction. Then the extract was resuspended in 180 µl lysis buffer GL and 25 µl proteinase K solution and vortexed vigorously. The mixture was incubated at 56˚C for 1 hour 30 minutes. The samples were then lysed by vortexing briefly for 3 minutes and adding 200 µl lysis buffer G3 then vortexed vigorously and incubated at 70˚C for 10 minutes. After the incubation the extracts were vortexed briefly and 200 µl of ethanol (96% - 100%) was added to the sample followed by a vigorous vortexing. Then for each sample it was placed in ISOLATE II Genomic DNA spin column into a collection tube. The entire sample was added to the column and centrifuged for 1 minute at 11,000 gravity to bind the total DNA. The flow through was discarded and each of the collection tube reused as per the kit instructions. The centrifugation was repeated at a higher gravity force for those whose samples had not completely filtered through the matrix. Then 500 µl wash buffer GW1 was added and centrifuged for 1 minute at 11,000 gravity at 25˚C. The flow through was discarded and the collection tube reused. This was followed by addition of 600 µl wash buffer GW2 to the column and centrifugation for 1 minute at 11,000 gravity. The flow through was discarded and collection tube reused. The resultant product was centrifuged at 11,000 gravity to remove residual ethanol and placed the ISOLATE II Genomic DNA spin column in a 1.5 ml micro-centrifuge tube. Finally, the DNA was eluted by adding 30 µl of preheated elution buffer G at 70˚C directly onto si-

lica membrane and incubated at room temperature for 1 minute. The samples were then centrifuged at 11,000 gravity for 1 minute then repeated by repassing the 30 µl through the silica membrane; centrifuging again before finally topping up the final volume to 60 µl to ensure the limited *Ustilago kamerunensis* DNA was not diluted but concentrated.

3.4. Amplification of *Ustilago kamerunensis* Isolates Genomic DNA

The isolated DNA samples with a negative control (has no DNA sample put in this treatment but other PCR reaction reagents are involved) was amplified using a modified methodology [39]. The ITS1-ITS4 primer pair whose sequences are shown on Table 1 were used to amplify the intervening 5.8S rDNA, and the adjacent ITS1 and ITS2 regions. PCR amplification was performed with a volume of 50 µl. Two microliters of each sample was added to the PCR master mixture, which consisted of 5 µl of 10× PCR buffer, 4 µl of a deoxynucleoside triphosphate mixture (0.1 mM each dNTP), 0.8 µl of each primer (40 pmol of each primer), and 0.4 µl (2.0 U) of Ex*Taq* DNA polymerase (Takara Biomedicals, Osaka, Japan), with the remaining volume consisting of distilled water. Amplification consisted of an initial denaturation at 94˚C for 4 minutes; 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 55˚C for 30 seconds, and extension at 72˚C for 1 minute; and a final extension at 72˚C for 4 minutes; a GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer Corp., Emeryville, Calif.) was used. Negative control reactions without any template DNA were carried out simultaneously. Amplified products were separated on 1.5% agarose gels in 1× TBE buffer at 10 $V \cdot cm^{-1}$ for 30 minutes. Amplification products were then stained with ethidium bromide and observed with a BioRad UV transilluminator. After the gel was photographed, the bands were located by using UV lamp, cut out and placed in a 2 ml eppendorf. The PCR fragments were then extracted from the gel using Qiagen Gel extraction kit protocol.

3.5. Sequencing and Phylogenetic Analysis of the Head Smuts' Amplified Sequences

Sequencing and phylogenetic analysis was done using the *Ustilago kamerunensis* isolates' polymerase chain reaction products using a modified methodology [33]. The products exhibiting the clear bands under the UV trans-illuminator were purified for sequencing using Qiagen kit as per the manufacturer's protocol (QIAGEN Inc., Valencia, CA). Five volumes of binding buffer (BB) was added to one volume of PCR products (100 µl to 20 µl) and transferred to Qia-quick

Table 1. Primer pairs used to amplify the *Ustilago kamerunensis* isolates genomes.

Primer type	Primer sequences
Internal Transcribed Spacer Primers	ITS 1; 5'-TCCGTAGGTGAACCTGCGG-3'
	ITS 4; 5'-TCCTCCGCTTATTGATATGC-3'

Source: [40].

column in provided 2 ml collection tube. The samples were introduced into the column and spinned for 1 minute. The flow through was discarded and the column returned back in the same tube. Then 0.7 ml wash buffer (PE) was added to the Qia-quick column and spinned for 1 minute at 13,000 rpm. The flow through was discarded and placed back to the column in the collection tubes. A short spin was performed to remove residual wash buffer. The columns were then placed in clean 1.5 ml microcentrifuge tube, 30 µl elution buffer (buffer EB) or molecular grade water (pH7) was added to elute DNA and spinned for 1 minute at 13,000 rpm. The eluted DNA was used for sequencing at Bioneer laboratory, South Korea. Sequencing reactions were performed using the BigDye Terminator v3.1 sequencing Kit (Applied Biosystems, USA) with the primers ITS1-F, and ITS4-R. 12 µl of (4µl ss DNA, 2 µg, 4 µl, 0.8 µM primer, 2 µl 10× MOPS buffer and 2 µl 10× Mn[2+] isocitrate buffer) was added in 1.5ml microcentrifuge tube, then incubated at 65˚C - 70˚C for 5 minutes to denature DNA and allow primers anneal. The reaction was allowed to cool at room temperature for 15 minutes, and then briefly centrifuged to reclaim condensation. To each reaction, 22 µl (7 µl ABI terminator mix (401489), 2 µl diluted Sequenase [TM] (3.25 U/µl), and 1 µl 2 mM a-S dNTPs) was added and incubated for 10 minutes at 37˚C before 20 µl 9.5 M ammonium acetate and 100 µl 95% ethanol was added and vortexed. It was then centrifuged again for 15 minutes, and carefully the supernatant decanted. DNA was then precipitated in ice-water bath for 10 minutes, centrifuged for 5 minutes at 12,000 rpm in a microcentrifuge at 40˚C and supernatant carefully decanted and rinsed in 300 µl of 70% - 80% ethanol. DNA was then dried for 5 - 10 minutes in the Speedy-Vac. Thermal-cycling Conditions included 60˚C for 30 minutes and holding at 40˚C. Sequenced products were analyzed in an automatic sequencer, ABI3730XL (Applied Biosystems).

3.6. Statistical Analysis

The analysis of variance of the geographical positioning system (GPS) and *Ustilago kamerunensis* isolates growth *in vitro* data was analyzed using the PASW statistical software version 20. The obtained sequences from the amplified internal transcribed spacer regions were edited in chromas lite to remove the ambiguous bases. They were then subjected to BioEdit version 7 to generate consensus sequences from the forward and reverse primer fragments [41]. The in-house python script was used on the non-nucleotide characters from the fasta sequences before the sequences were submitted to National Center of Biotechnology Information (NCBI) GenBank. The nucleotide alignment was performed by CLUSTAL W that was implemented in BioEdit version 7 upon trimming the ends [16]. The gaps in the alignment were deleted by online program Gap Strip/Squeeze version 2.1.0 only allowing 20% gap tolerance. The alignment file was then loaded in MEGA 7 where evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [42]. Initial trees for the heuristic search were obtained automatically by applying Neigh-

bor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 17 nucleotide sequences. Codon positions included were 1st, 2nd, 3rd and non-coding. All positions containing gaps and missing data were eliminated. There were a total of 252 positions in the final dataset [43]. In estimating evolutionary divergence between Sequences, analyses were conducted using the Jukes-Cantor model [44]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 17 nucleotide sequences and codon positions included were 1st, 2nd, 3rd and non-coding. All positions containing gaps and missing data were eliminated. There were a total of 252 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [43] [44] [45].

4. Results and Discussion

4.1. Collection of *Ustilago kamerunensis* Isolates

The molecular characterization of the napier head smut disease causing agent was preceded by collection of eighteen *Ustilago kamerunensis* isolates from eighteen hot spot areas within the five counties of Central Kenya region and Nakuru county totaling to six areas as demonstrated on **Table 2** and **Table 3**. The collection of *Ustilago kamerunensis* isolates from Nakuru county confirmed the report about the region being the newly affected zone as the pathogen continues to spread westwards of Central Kenya region towards the high altitude areas of the Rift-valley [10]. The mean ± standard error of the points of collection's altitude levels was 1988.17 ± 71.97 metres above sea level (**Table 3**). Isolate NYA003 (NYA-3) of Nyandarua county was collected from the highest altitude levels (2698 m above sea level), followed by NYA002 (NYA-2), NAK002 (NAK-2) and NYA001 (NYA-1) isolates that were collected from 2440, 2268 and 2240 metres respectively. KIR009 (KIR-9) isolate was collected from the lowest altitude (1344 m) followed by KIR002 (KIR-2) and KIR001 (KIR-1) that were collected from 1693 and 1699 metres respectively (**Table 2**). In the general evaluation of the mean altitudes of the isolate collection points across the counties, significant differences were observed at ($df = 5$; $F = 9.81$; $P = 0.001$). This confirmed an earlier report on napier head smut occurrence, which noted that the disease's hot spot areas altitude level ranged between 1660 to 2400 metres above sea level [30]. Additionally, when comparing the counties of collection Nyandarau county had the highest altitude mean in isolate collection points, followed by Nakuru county which was an intermediate performer though not statistically different from the mean of Nyandarua county (**Table 3**). Kirinyaga county exhibited the lowest altitudes with the rest of the counties exhibiting intermediate levels of altitude in collection points of the isolates (**Table 3**). The effect of altitude on the disease is further supported by an observation, where during the collection of the eighteen isolates no head smut affected napier grass was observed in the low altitude zones of the Nakuru county; that is areas below the

Table 2. The eighteen *Ustilago kamerunensis* isolates collected from different affected counties' hot spot areas of Central Kenya showing the respective co-ordinates and altitude levels.

No.	*Ustilago kamerunensis* Isolates' GenBank codes	Synonyms or Short forms	County of Origin	Collection Point Co-ordinates and Altitudes		
				Latitude	Longitude	Altitude
1.	MUR001	MUR-1	Murang'a	S 00.81380˚	E 037.03799˚	1931 m
2.	MUR002	MUR-2	Murang'a	S 00.72882˚	E 036.87831˚	2102 m
3.	MUR003	MUR-3	Murang'a	S 00.68270˚	E 036.90532˚	1935 m
4.	KIA001	KIA-1	Kiambu	S 01.18049˚	E 036.64774˚	2157 m
5.	KIA002	KIA-2	Kiambu	S 01.17762˚	E 036.74564˚	1928 m
6.	KIA003	KIA-3	Kiambu	S 01.08774˚	E 036.78181˚	1871 m
7.	NYA001	NYA-1	Nyandarua	S 00.49860˚	E 036.48170˚	2240 m
8.	NYA002	NYA-2	Nyandarua	S 00.40371˚	E 036.49390˚	2440 m
9.	NYA003	NYA-3	Nyandarua	S 00.87156˚	E 036.57031˚	2698 m
10.	NAK001	NAK-1	Nakuru	N 00.00876˚	E 036.25268˚	2063 m
11.	NAK002	NAK-2	Nakuru	S 00.02934˚	E 036.20500˚	2268 m
12.	NAK003	NAK-3	Nakuru	S 00.17176˚	E 036.12392˚	1929 m
13.	KIR001	KIR-1	Kirinyaga	S 00.47394˚	E 037.22716˚	1699 m
14.	KIR002	KIR-2	Kirinyaga	S 00.37333˚	E 037.30536˚	1693 m
15.	KIR009	KIR-9	Kirinyaga	S 00.54285˚	E 037.30877˚	1344 m
16.	NYE001	NYE-1	Nyeri	S 00.58910˚	E 036.95040˚	1817 m
17.	NYE002	NYE-2	Nyeri	S 00.46589˚	E 036.94195˚	1844 m
18.	NYE004	NYE- 4	Nyeri	S 00.37537˚	E 036.93737˚	1828 m

Table 3. The general evaluation of the altitude levels' means across the counties where respective isolates were collected.

County of Collection	*Ustilago kamerunensis* Isolates' Points of Collection Mean Altitudes in Metres ± S.E
Nyandarua County	2459.33 ± 132.57 a
Nakuru County	2086.67 ± 98.57 ab
Murang'a County	1989.33 ± 56.35 bc
Kiambu County	1985.33 ± 87.40 bc
Nyeri County	1829.67 ± 7.84 bc
Kirinyaga County	1578.67 ± 117.35 c
Mean altitude ± S.E	**1988.17 ± 71.97**
Test values	**df = 5; F = 9.81; P = 0.001**

Mean altitude levels (m) of the *Ustilago kamerunensis* isolates collection points ± standard error of each county. Means having the same letters in the same column do not differ significantly from each other at $p > 0.05$. Those with more than one letter within a column are intermediates in performance.

reported altitude range of 1660 to 2400 metres above sea level [30]. This suggested that the disease's occurrence and infectivity might be influenced by a relatively high altitude range. Therefore, making the high altitude areas to be at a

greater risk of perpetuating the disease's spread across the country. This proposition is further supported by new reports of the disease around Molo area; another high altitude zone of the Rift-valley [1]-[10]. The Molo area is separated to the west of the collection points of the Nakuru county isolates (NAK001, NAK002 & NAK003) that were characterized in this study by a low altitude region where the disease was not observed (**Figure 1**).

4.2. Mapping of the *Ustilago kamerunensis* Isolates Distribution Basing on Collection Points

The coordinates of the points of collection were used to generate a map shown on **Figure 1**; showing the hotspot areas of the affected regions/counties in Kenya, where the respective eighteen isolates' ustilospores were collected. As observed from the map the *Ustilago kamerunensis* isolates were evenly distributed across the five Central Kenya counties. However, in Nakuru county only the high altitude northern parts of the county exhibited the disease unlike the low altitude parts where the Rift-Valley stretches. Moreover, the eastern side of the Nakuru county bordering Central Kenya was the one that seemed affected by

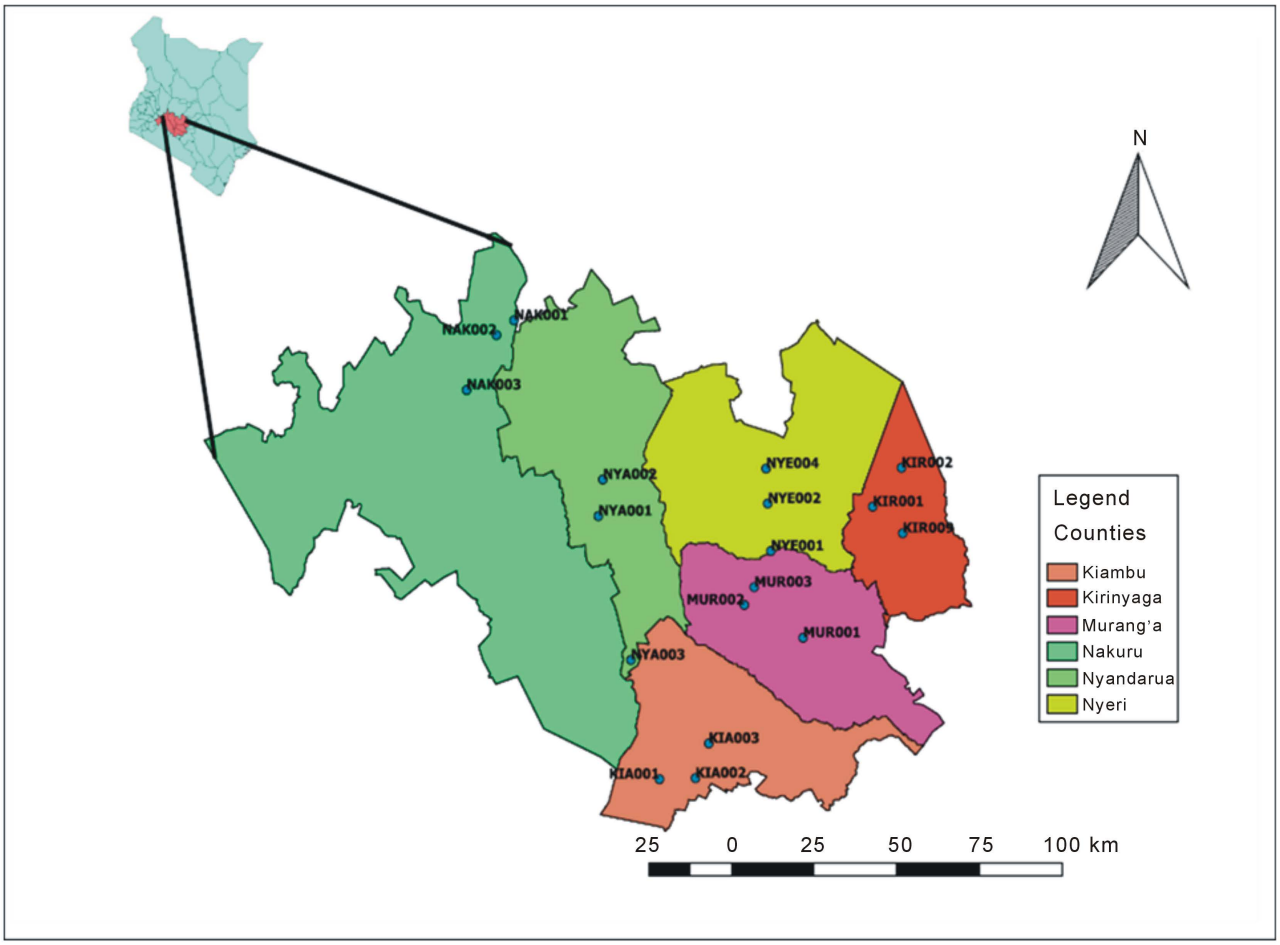


Figure 1. A generated map showing the surveyed six affected counties in Central part of Kenya and the napier head smut hot spot areas where respective *Ustilago kamerunensis* isolates were collected from for molecular characterization.

the disease unlike the western side. The density of the black ustilospores on affected napier grass inflorescence seemed low, scattered and less severe on the infected napier grass tillers in Kirinyaga county's hot spot areas unlike the other counties surveyed where the density was quite high (**Figure 2**). This observation seemed to support the propositions that this napier head smut disease agent, seems to be more infectious in high altitude areas above 1660 masl [30]. This is because the areas with slightly lower altitude levels like Kirinyaga county (**Table 3**), the disease was scarce and the few infected napier grass tillers' inflorescences that were found exhibited a low density of the smut ustilospores (**Figure 2**). Moreover, the collection of isolate KIR009 (KIR-9) whose 009 code denotes its collection being from an area whose incidence was below 20% seems to support the less severe nature of the disease in low altitude areas. A scenario which was not observed in high altitude hot spot areas whose inflorescences had a high density of the black ustilospores. This phenomenon can be attributed to several interacting factors that influence the pathogen and the host napier grass varieties. For instance the effect of altitude on some elements of weather like temperature, humidity and precipitation, which in turn might be affecting the pathogen establishment [39]-[46]. The altitude increase leads to temperature decrease translating to high levels of condensation that end up varying the presence of free water which it interacts with and is very critical in completing the disease's triangle [47] [48]. A similar scenario has been observed in other smut diseases' infection triangle where cool, humid and wet conditions typical of high altitude areas enhanced the establishment of the fungal pathogens like; karnal bunt

Figure 2. Highly magnified images of four different napier grass inflorescences labelled in a clockwise manner from image (a), showing different densities of the black ustilospores. Images (a), (d) on the left and (b), (c) on the right exhibit low and high density of ustilospores respectively.

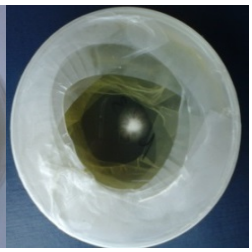
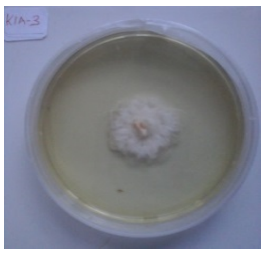
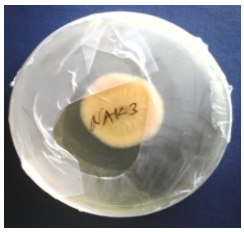
(*Tilletia indica*), flug smut (*Urocystis agropyri*) and the onion smut (*Urocystis cepulae*) [3] [49] [50] [51]. According to the reports on the onion smut, the pathogen attacks severely the onions on the northern high altitude cold areas of the USA and Europe, whereas the southern low altitude warm areas of the USA despite growing onions are smut free, a trend similar to the one observed on napier head smut pathogen (*Ustilago kamerunensis*) infection pattern in Kenya [51]. Additionally, the low temperature associated with high altitude areas has been reported to optimally stimulate sclerotial formations on some of these smuts [50].

Another, dimension can be the possible modification of the chemical composition of the host napier grass varieties by their location, season , genotype and management practices during their growth which has been reported to influence their crude protein levels and cell structure in various studies [52] [53]. The modification of the host chemical composition in turn alters the levels of suitability of the host to the pathogen as observed in a typical disease triangle and possibly increasing the napier grass varieties susceptibility in high altitude areas, especially if the disease resistance trait of napier grass varieties is a polygenic type which is highly unstable and influenced by environment due to the interaction of the many genes that express such traits [54] [55]. Additionally, the localities' temperature differences is known to influence highly the physiology of plants; where under relatively warm temperatures the plants tend to translocate photosynthates rapidly leading to enhanced vigour in growth unlike areas that experience low temperatures synonymous with high altitude areas. Hence, possibly explaining the observed high susceptibility of napier grass germplasm in high altitude areas [56] [57].

4.3. Laboratory Evaluation of *Ustilago kamerunensis* Isolates Growth *In Vitro*

Focusing on the laboratory evaluations of the collected isolates' ustilospores growth *in vitro* on malt extract agar, some easily distinguishable morphological differences in colony size were observed at day four of culture. Basing on the colony colour, all of them exhibited predominantly white floccose colonies on top side and pale cream at the reverse (**Table 4**). This confirmed the findings by [30]-[58]. In the colony diameters' evaluation, significant differences at ($df = 17$; $F = 52.22$; $P \leq 0.0001$) and ($df = 17$; $F = 321.88$; $P \leq 0.0001$) were observed for both trials one and two respectively (**Table 5**). In trial one NAK002 isolate from Nakuru county led in growth vigour with the largest colony diameter, followed by NYA003 and KIA001 whose colony diameters were not statistically different in second and third position respectively. The NYA002 isolate exhibited the smallest colony diameter in this trial followed by NAK003 isolate as the other isolates performed intermediately (**Table 5**). During the trial two evaluation NAK002 isolate still retained the lead with the largest colony diameter followed by; NAK003, MUR003, MUR001 and MUR002 in that order though their colony

Table 4. Pictures of the morphological variations observed during growth *in vitro* on malt extract agar of selected four *Ustilago kamerunensis* isolates that exhibited easily distinguishable characteristics especially on the colony size at day four of culture.

***Ustilago kamerunensis* Isolates**	**NAK003 (NAK-3) Isolate**	**NAK002 (NAK-2) Isolate**	**NYA003 (NYA-3) Isolate**	**KIA003 (KIA-3) Isolate**
Top-side Colony Picture				
Reverse-side Colony Picture				

The colonies had been cultured at 25˚C and colony diameter measured on day four which is the recommended culture period for fungal cultures for clear observations. It is important to note the pictures on this table are not presented to scale, but are aimed at demonstrating the morphological differences observed.

Table 5. Comparative analysis between two trials of *in vitro* growth of the *Ustilago kamerunensis* isolates' on malt extract agar.

***Ustilago kamerunensis* Isolates**	**Mean Growth Diameter (Trial one)**	**Mean Growth Diameter (Trial two)**
NAK002 (NAK-2)	3.64 ± 0.34 a	3.56 ± 0.20 a
NYA003 (NYA-3)	2.34 ± 0.27 b	1.42 ± 0.10 c
KIA001 (KIA-1)	2.19 ± 0.22 b	1.45 ± 0.15 c
KIA002 (KIA-2)	2.01 ± 0.51 bc	1.53 ± 0.14 c
KIA003 (KIA-3)	1.99 ± 0.41 bc	1.39 ± 0.07 c
KIR002 (KIR-2)	1.88 ± 0.09 cd	1.48 ± 0.09 c
NAK001 (NAK-1)	1.87 ± 0.29 cd	2.55 ± 0.15 c
NYA001 (NYA-1)	1.85 ± 0.12 cd	1.41 ± 0.16 c
NYE002 (NYE-2)	1.72 ± 0.21 def	1.42 ± 0.10 c
NYE004 (NYE-4)	1.69 ± 0.10 def	1.44 ± 0.12 c
MUR003 (MUR-3)	1.66 ± 0.05 def	2.49 ± 0.07 b
MUR001 (MUR-1)	1.65 ± 0.23 def	2.44 ± 0.12 b
KIR009 (KIR-9)	1.64 ± 0.04 def	1.44 ± 0.08 c
KIR001 (KIR-1)	1.64 ± 0.08 def	1.47 ± 0.14 c
NYE001 (NYE-1)	1.63 ± 0.07 def	1.43 ± 0.08 c
MUR002 (MUR-2)	1.55 ± 0.27 ef	2.41 ± 0.07 b
NAK003 (NAK-3)	1.34 ± 0.22 fg	2.55 ± 0.19 b
NYA002 (NYA-2)	1.14 ± 0.13 g	1.38 ± 0.08 c

Mean growth diameter (cm) of the *Ustilago kamerunensis* isolates ± standard deviation. Means having the same letters in the same column do not differ significantly from each other at $p > 0.05$. Those with more than one letter within a column are intermediates.

diameter means did not exhibit statistical differences. The NYA002 and KIA003 isolates had the lowest and second lowest colony diameter respectively, despite not exhibiting statistical differences with many of the other isolates which had smaller colony diameter means in the second trial (Table 5). In the comparative ranking across the two trials; the NAK002 isolate led with the largest colony diameter whereas NYA002 isolate exhibited the smallest diameter (Table 6). These differences in growth *in vitro* are typical of physiologic isolates and has been observed in many microbial species under culture in relation to their growth kinetics. A scenario that can be due to the microbes varying adaptability to their ecological niches brought about by agents of evolution in their areas of occurrence [59] [60] [61]. These variations in growth *in vitro* are largely influenced by the way each isolate has adapted its physiology to survive varying water activity levels and other physiochemical conditions associated with media during culture that leads to different physiologic responses. This strategy has been used as the first line of selection towards identification of physiologic races of a particular pathogen before evaluation using differential cultivars [62]. Also, such physiologic differences have enabled biological characterization and selection of biological control agents [35].

Table 6. Comparative ranking of the respective *Ustilago kamerunensis* isolates' basing on their overall performance in growth *in vitro* as per the two trials evaluated.

Ustilago kamerunensis Isolates	*In vitro* Growth Performance Trial one (Rank)	*In vitro* Growth Performance Trial two (Rank)	*In vitro* Growth Average Performance (Overall rank)
NAK002 (NAK-2)	1	1	1
NYA003 (NYA-3)	2	3	3
KIA001 (KIA-1)	2	3	3
KIA002 (KIA-2)	3	3	3
KIA003 (KIA-3)	3	3	3
KIR002 (KIR-2)	4	3	4
NAK001 (NAK-1)	4	3	4
NYA001 (NYA-1)	4	3	4
NYE002 (NYE-2)	5	3	4
NYE004 (NYE-4)	5	3	4
MUR003 (MUR-3)	5	2	4
MUR001 (MUR-1)	5	2	4
KIR009 (KIR-9)	5	3	4
KIR001 (KIR-1)	5	3	4
NYE001 (NYE-1)	5	3	4
MUR002 (MUR-2)	6	2	4
NAK003 (NAK-3)	7	2	5
NYA002 (NYA-2)	8	3	6

Mean performance rank was determined by getting the average rank of a respective isolate basing on its respective ranking in the two trials.

4.4. Molecular Analysis of *Ustilago kamerunensis* Isolates Sequenced Regions and Submission to GenBank

The eighteen *Ustilago kamerunensis* isolates total DNA was extracted and analyzed. The PCR fragment of about 600 base pairs (**Figure 3**) was obtained from the sampled first 9 sequences. This was expected and therefore it was satisfactory that the required region was successfully amplified. The **Figure 4** shows the alignment file, where conserved loci were observed at different positions within the ITS region and many more as shown by the uniform colouring of some regions. Nucleotide substitution and deletions were observed at certain positions of some of the *Ustilago kamerunensis* isolates' nucleic acid sequences comparatively (**Figure 4**). The sequences of the seventeen (17) *Ustilago kamerunensis* shown on the far left of the **Figure 4** were submitted at the National Center for Biotechnology Information GenBank. Seventeen because the DNA quality and quantity of isolate NYA003 (NYA-3) was not good and extremely low to support sequencing procedure. Further, of the seventeen that were sequenced; one isolate's sequence that is MUR001 did not merit passing the NCBI filters and therefore was not assigned an accession number. That meant out of 17 sequences, 16 were assigned accession numbers which they can be accessed by from the GenBank database as shown on **Table 7**. The isolates sequences were

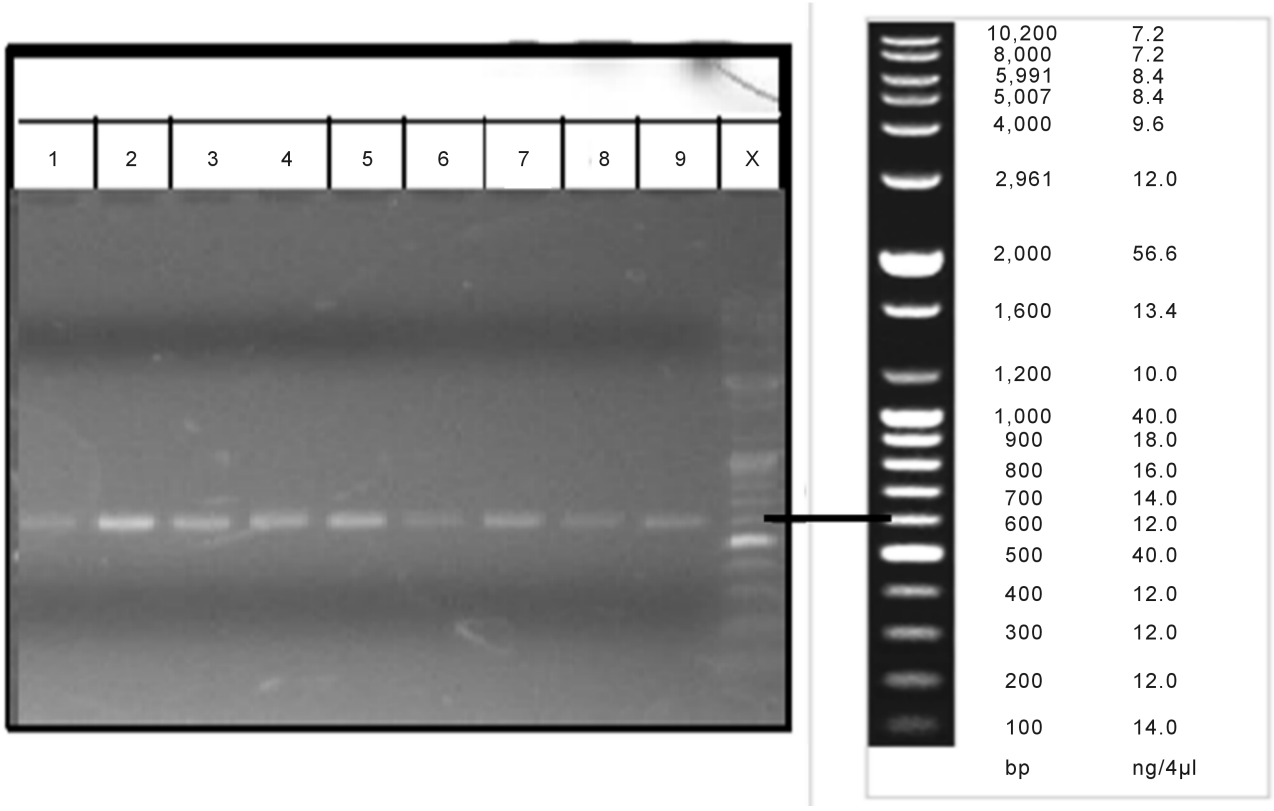


Figure 3. An electropherogram of the sample nine PCR products. X is the ladder, showing the DNA bands at approximately 600 bp (base pairs) level of *Ustilago kamerunensis* visualized upon amplification of the total DNA using ITS primers towards sequencing. The ladder used is DNA-plus ladder developed by Bioneer.

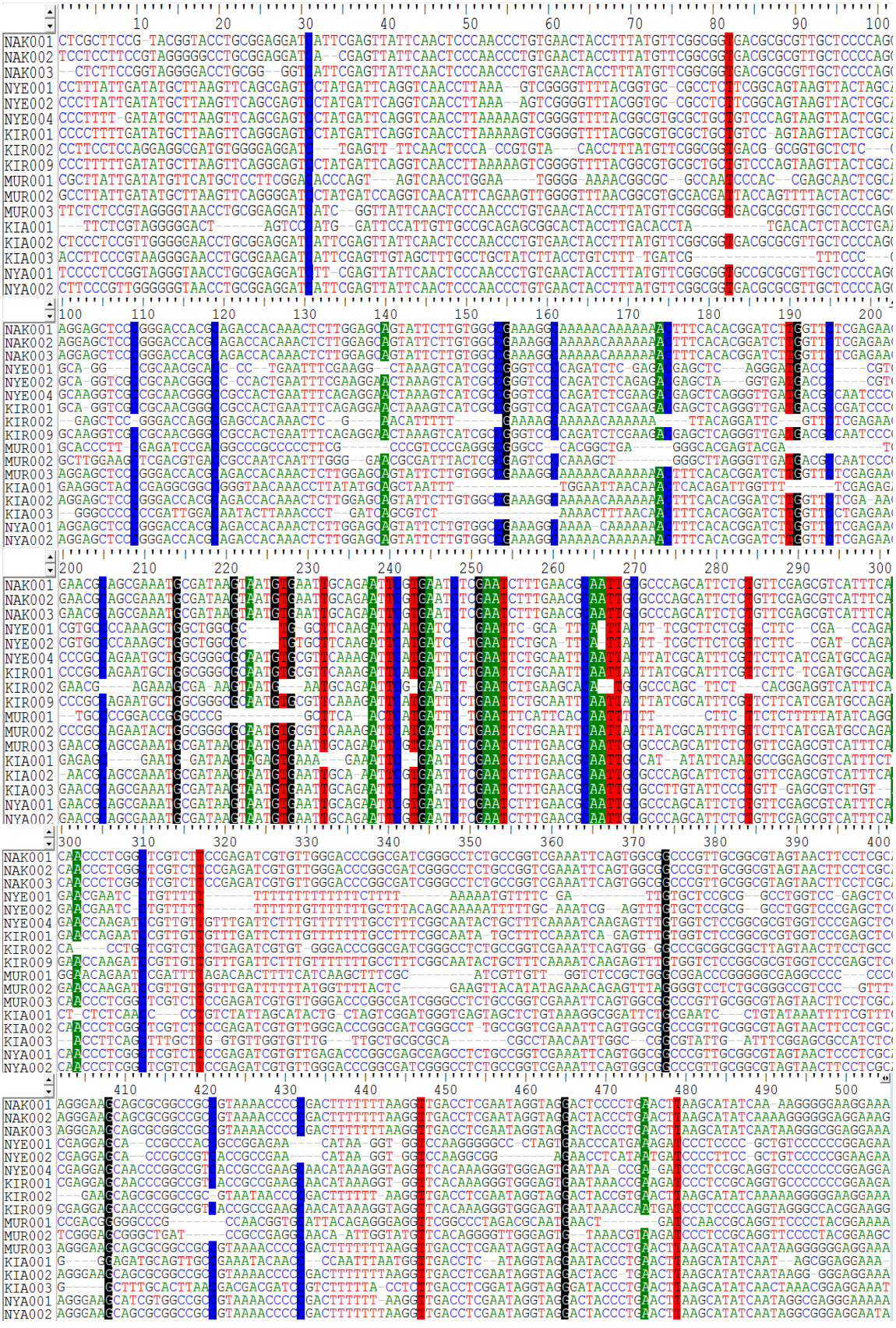


Figure 4. Sequence alignments as viewed in Bioedit version 7. The blocks with the same colour indicate conserved loci.

Table 7. National Center for Biotechnology Information's (NCBI) GenBank accession numbers of the sample isolates up on submission. Searching the accession number at GenBank's domain the details of each isolate's molecular structure is availed.

Sample	Organism name	Strain	Accession number
SUB3393968 NAK001	*Ustilago kamerunensis*	NAK001	MG722754
SUB3393968 NAK002	*Ustilago kamerunensis*	NAK002	MG722755
SUB3393968 NAK003	*Ustilago kamerunensis*	NAK003	MG722756
SUB3393968 NYE001	*Ustilago kamerunensis*	NYE001	MG722757
SUB3393968 NYE002	*Ustilago kamerunensis*	NYE002	MG722758
SUB3393968 NYE004	*Ustilago kamerunensis*	NYE004	MG722759
SUB3393968 KIR001	*Ustilago kamerunensis*	KIR001	MG722760
SUB3393968 KIR002	*Ustilago kamerunensis*	KIR002	MG722761
SUB3393968 KIR009	*Ustilago kamerunensis*	KIR009	MG722762
SUB3393968 MUR001	*Ustilago kamerunensis*	MUR001	NA
SUB3393968 MUR002	*Ustilago kamerunensis*	MUR002	MG722763
SUB3393968 MUR003	*Ustilago kamerunensis*	MUR003	MG722764
SUB3393968 KIA001	*Ustilago kamerunensis*	KIA001	MG722765
SUB3393968 KIA002	*Ustilago kamerunensis*	KIA002	MG722766
SUB3393968 KIA003	*Ustilago kamerunensis*	KIA003	MG722767
SUB3393968 NYA001	*Ustilago kamerunensis*	NYA001	MG722768
SUB3393968 NYA002	*Ustilago kamerunensis*	NYA002	MG722769

The SUB3393968 MUR001 strain was not assigned an accession number by GenBank, because it lacked some genome elements that merit the assigning during the filtering process subjected to the sequences by the database management system. The initials "NA" denote "Not Assigned" an accession number.

then subjected to a phylogeny analysis to determine the degree of divergence from the ancenstral stock as shown on **Figure 5**. The tree with the highest log likelihood (−2319.75) is shown in **Figure 5**. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All sequences from Nakuru, Nyahururu and Kiambu counties were put in cluster A. Cluster B contained sequences from Nyeri, Kirinyaga and Murang'a. One of the sequences from Murang'a (MUR003) and Kirinyaga (KIR002) were classified in Cluster A (**Figure 5**). This could be due to the susceptible napier grass germplasm transfer by farmers, where they unknowingly exchange cuttings containing the different isolates within the region leading to their introduction in other areas in an irregular manner [1] [11]-[63]. Samples from Nakuru, Nyahururu as well as those from Nyeri appeared very phylogenetically similar as they formed respective sub-clusters. All sequences from Nakuru, Nyahururu and Kiambu counties formed a common clade, whereas, the isolates from Nyeri, Kirinyaga and Murang'a formed the other (**Figure 5**). Giving a likely indication of wind transmission of the smut spores northwards in a regular pattern now that

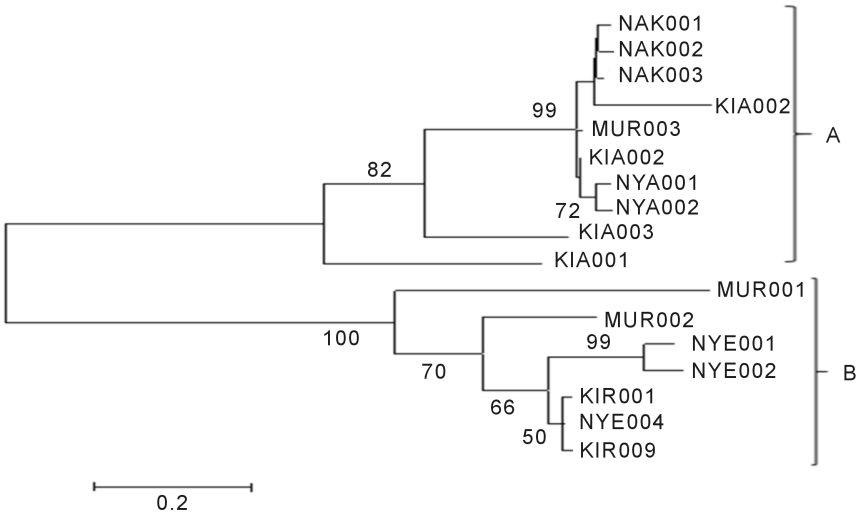


Figure 5. A phylogenetic tree based on the molecular phylogenetic analysis by Maximum Likelihood method. The ITS sequences of the different *Ustilago kamerunensis* isolates from affected counties of Kenya were used in the assessment.

the counties mentioned are next to each other in northly manner as shown on **Figure 1**. Wind has been identified as another mode of transmission of the napier head smut disease caused by *Ustilago kamerunensis* [29] [30] [31].

The number of base substitutions per site between sequences are shown on **Table 8**. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). The pairwise evolutionary divergence (**Table 8**) showed that the sequences had very high genetic diversity because no sequences had genetic distance zero. In fact the overall genetic distance of all the samples in the experiment was 2.249 ± 0.670. This value is so high and therefore this is an indication of high genetic diversity among the *Ustilago kamerunensis*. The strains with the lowest genetic distance was between NYA001 and NYA002 (0.042 ± 0.014) while the highest genetic distance of 5.693 ± 3.516 and 5.693 ± 4.085 was observed between the strains (KIA001 versus MUR002) and (KIR002 versus NYE002) respectively. Generally most of the samples demonstrated a high genetic diversity (**Table 8**). Also, the statistical confidence levels of placing the different clades on their nodes by the bootstrap procedure was very high as shown on **Figure 5**. The molecular differences observed in the isolates of *Ustilago kamerunensis* could be due to environmental restrictions of the pathogen, especially by altitude [30]. Coupled to that is the introduction of tolerant napier grass varieties in Central Kenya like Kakamega 1 and 2, has subjected the pathogen to a lot of environmental selection pressure. A situation that triggers rapid selection and changes in a pathogen in effort to survive the threats [64]. Generally the smut pathogens have been reported to display rapid changes in their genes through co-evolution especially those involved in the coding for effector proteins towards improving their plant-pathogen interaction levels. A similar phenomenon has been observed in some fungal pathogens of wheat a poaceae like napier grass [26]-[65].

Table 8. The genetic distances matrix table showing the estimates of evolutionary divergence between Sequences of the different *Ustilago kamerunensis* isolates from affected Counties of Kenya.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. NAK001		0.012	0.011	2.148	2.251	1.230	1.148	0.034	0.989	3.988	2.281	0.015	0.162	0.014	0.083	0.019	0.017
2. NAK002	0.038		0.011	2.109	2.049	1.078	1.071	0.030	0.879	2.940	1.810	0.015	0.140	0.015	0.076	0.019	0.015
3. NAK003	0.029	0.033		1.841	1.669	0.961	0.950	0.031	0.795	2.656	2.115	0.013	0.160	0.013	0.086	0.018	0.018
4. NYE001	4.500	4.312	4.138		0.022	0.037	0.040	4.865	0.039	0.159	0.086	2.502	3.421	2.767	2.451	1.862	2.019
5. NYE002	4.500	4.312	3.975	0.094		0.037	0.039	4.085	0.039	0.189	0.087	2.080	2.614	2.247	1.203	1.301	1.996
6. NYE004	3.545	3.300	3.081	0.195	0.208		0.008	2.078	0.009	0.160	0.049	1.072	1.291	1.167	0.918	0.945	0.977
7. KIR001	3.419	3.300	3.081	0.214	0.228	0.016		1.946	0.011	0.150	0.052	1.071	1.275	1.032	0.907	0.853	0.879
8. KIR002	0.189	0.176	0.183	6.000	5.693	4.312	4.312		1.529	4.620	2.498	0.034	0.166	0.034	0.115	0.041	0.035
9. KIR009	3.187	2.980	2.794	0.214	0.228	0.020	0.029	3.823		0.165	0.050	0.885	1.109	0.944	0.870	0.892	0.929
10. MUR001	5.413	4.702	4.702	0.938	1.091	0.938	0.898	5.413	0.958		0.163	2.240	3.516	3.606	3.413	2.558	2.638
11. MUR002	4.920	4.312	4.702	0.516	0.539	0.292	0.308	4.920	0.300	0.938		2.084	3.456	2.277	0.759	1.602	1.700
12. MUR003	0.051	0.055	0.042	4.500	4.312	3.300	3.300	0.195	2.980	4.500	4.500		0.140	0.006	0.075	0.014	0.014
13. KIA001	1.000	0.879	0.979	5.156	4.702	3.545	3.545	1.000	3.300	5.693	5.156	0.879		0.1440	0.137	0.167	0.149
14. KIA002	0.042	0.051	0.038	4.702	4.500	3.419	3.187	0.195	3.081	5.156	4.702	0.012	0.898		0.073	0.014	0.014
15. KIA003	0.550	0.493	0.550	4.500	3.300	3.081	3.081	0.742	2.980	5.156	2.707	0.493	0.843	0.483		0.079	0.077
16. NYA001	0.074	0.069	0.065	4.138	3.680	3.081	2.885	0.248	2.980	4.500	3.975	0.046	1.000	0.042	0.516		0.014
17. NYA002	0.060	0.046	0.065	4.312	4.312	3.187	2.980	0.208	3.081	4.702	4.138	0.046	0.918	0.042	0.493	0.042	

The upper blue triangle values are showing the margin of error of the genetic distance between pairwise comparisons of isolates. Whereas, the lower black triangle values demonstrate the genetic distance levels between pairwise comparisons of *Ustilago kamerunensis* isolates.

5. Conclusion

This observed trend in Central Kenya on *Ustilago kamerunensis* isolates is worrying, as much as the sequenced regions might not be involved directly in their pathogenicity and virulence. There is a clear indication that molecular differences exist among the *Ustilago kamerunensis* isolates and probably extend to their virulence genes. Hence, over time due to environmental pressure and related dynamics, a highly virulent napier head smut pathogen which might not be limited by altitude differences might emerge, leading to massive losses. Further, the introduction of tolerant cultivars in the affected region should be done in a heterogeneous manner. The strategies of management need to be given much focus to ensure co-evolution rate is slowed especially through integrated pathogen management approaches.

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Conflict of Interest Declaration

The authors declare that there is no conflict of interest regarding the publication of this paper.

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