

GENETIC VARIABILITY CHARACTERIZATION OF ALUPE NAPIER GRASS ACCESSIONS**BASED ON SIMPLE SEQUENCE REPEAT MARKERS****ABSTRACT**

Genetic variability of 23 Napier grass clones, 22 of which were collected from Kenya Agricultural Research Institute – at Alupe in Kenya was determined using 17 simple sequence repeat (SSR) markers. The markers were polymorphic with the most discriminative being PSMP2267. Average gene diversity among the Napier grass clones was averaged at 0.54 with an average heterozygosity of 0.63. Total number of alleles across all loci was 90 with mean number of alleles per locus of 5.29 and a mean polymorphic information content of 0.50. Results also indicate a high genetic distance among the Napier grass clones with the most distant clone being 16814 while the closest was between kakamega1 and kakamega2, and kakamega2 and kakamega3. Principle component analysis did not group the clones in a definitive structure, with most clones scattered. However, the SSR markers grouped the clones into two clusters derived from Neighbour joining analysis with clone 16814 being alone in a cluster. Clone Kakamega1, Kakamega2 and Kakamega3 were too closely related. These findings are key in guiding selection of clones to include in the breeding programs and conservation of Napier grass.

Key words: Analysis, cluster, gene, heterozygosity, Neighbour, polymorphic

INTRODUCTION

Napier grass (*Pennisetum purpureum*), also known as elephant grass is a robust perennial forage indigenous to sub-Saharan Africa (1). The grass is dominant in the fertile crescent along north of Lake Victoria and the western Rift Valley in Uganda (2). Currently, Napier grass is the principal fodder crop in smallholder intensive and semi intensive livestock production systems in East Africa (3), constituting 40 – 80% of forages used to meet the increasing demand for milk. The demand for Napier grass is growing, mostly among poor households in densely populated areas due to its desirable traits such as tolerance to drought, ability to grow in a wide range of soil conditions, high photosynthetic and

water-use efficiency (4), withstand repeated cutting and rapid regeneration, producing a high yield that is very palatable to cattle in the leafy stage (1).

Napier grass productivity in the East African region is limited by several factors especially emerging new diseases like Napier Grass Stunt Disease and Napier Grass Head Smut Disease, thus constraining the growth of smallholder dairy industry (5). Therefore, continued utilization of Napier grass as a fodder will depend on exploitation of the genetic variability within and among its populations (6) in search for resistance. This requires a well characterized and inventoried germplasm; which is lacking in the case of Napier grass in East African countries such as Uganda (7). In East Africa Kenya Agricultural Research Institute – Dairy Research Centre at Alupe maintains a collection of *Pennisetum purpureum*; obtained from within Kenya and the International Livestock Research Institute, whose genetic diversity is not known.

Various methods for estimating diversity in a plant population exist and use of simple sequence repeats (SSRs) is the method of choice because of the markers multi-allelism, genome specificity, even distribution and high polymorphism. However, the genome of Napier grass has not been sequenced, therefore, Napier grass SSR markers are not known. The available option is through cross-amplification using SSR markers of closely related species (8). This study, therefore, determined the genetic variability among Napier grass clones maintained Kenya Agricultural Research Institute – Alupe station through cross- amplification using SSR markers of closely related organisms.

MATERIALS AND METHODS

Sample collection and analysis

Twenty two Napier grass clones obtained from Kenya Agricultural Research Institute – Dairy research Center Alupe and one clone - 16785 obtained from International Livestock Research Institute – Ethiopia were planted in the field at National Crops Resources Research Institute at Namulonge in Uganda in 6 replicates. Two months after planting, samples were collected from the inner most unfolded leaf on one tiller of each plant, placed in a paper bag with silica gels, packed in a box. These were transferred to Bioscience Eastern and Central Africa at International Livestock Research Institute (BecA-ILRI) - Nairobi for genotyping the same day.

55 In the laboratory, 1.5 g leaf sample was extracted from each sample and ground in mortar in liquid Nitrogen. Total plant
56 DNA was extracted using cetyltrimethylammonium bromide (CTAB) method (9) and diluted to 100μ using double
57 distilled water. The DNA concentration was determined using Nanodrop UV spectrometry at A260 and A280 while the
58 integrity of DNA was tested on 1.2% agarose gel electrophoresis in TBE buffer stained with gel red. From these,
59 template DNA was made from an aliquot in a 1.5 μl tube and diluted to 50ng/μ.

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61 DNA amplification with Microsatellite Markers

62 A total of 17 simple sequence repeat microsatellite primer pairs, originally identified in maize, pearl millet and sorghum
63 were conjugated with different dyes (VIC, NED, PET and 6-FAM). These were used in the PCR amplification in 20μl
64 *AccuPower® Taq* Premix (Bioneer) to which 17μl of water and 0.5μl of 5 picomoles of each of the primer pair and 2μl of
65 template DNA were added. The reaction mixture was subjected to the following PCR conditions: an initial denaturation
66 of 94°C (3min) followed by 35 cycles of 94°C (30sec); specific primer annealing temperature (1min) (Table 1); extension
67 at 72°C (2min), final extension at 72°C (10min) and final hold at 4°C. The PCR products were run on 1.2% agarose gel
68 electrophoresis stained with gel red in 0.5X TBE buffer at 80 V for 50 minutes and visualized on trans UV and
69 photographed in UVP DIGIDOC – IT system (UVP Bioluminescence systems, USA). The PCR products with clear single band
70 amplification on the agarose gel were subjected to capillary electrophoresis with ABI3730 DNA genetic analyser for
71 fragment analysis and allele calls were made using GENEMAPPER software v.3.7 (Applied Biosystems). Primers
72 whose PCR products generated high quality electropherogram peaks of fluorescent intensity above 50 at differing
73 positions in the samples were selected (Table 1) and used for amplification of all the samples.

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75 **Table 1. SSR primers used to assess genetic diversity in 23 Napier clones**

Primer Name	Sequence Left primer (Forward 5' – 3')	Right primer (reverse 5' – 3')	Annealing temperature (°C)
CTM-10	GAGGCAAAAGTGGAAGACAG	TTGATTCCCGGTTCTATCGA	52
CTM-27	GTTGCAAGCAGGAGTAGATCGA	CGCTCTGTAGGTTGAACTCCTT	52
CTM-59	TCCTCGACATCCTCCA	GACACCTCGTAGCACTCC	54
CTM-8	GCTGCATCGGAGATAGGGAA	CTCAGCAAGCACGCTGCTCT	52
PGIRD2	GCTATTGCCACTGCTTCACA	CCACCATGCAACAGCAATAA	54

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PGIRD2 5	CGGAGCTCCTATCATTCCAA	GCAAGCCACAAGCCTATCTC	58
PGIRD5 7	GGCCCCAAGTAACTTCCCTA	TCAAGCTAGGGCCAATGTCT	56
PSMP22 35	GCTTTTCTGCTTCTCCGTAGAC	CCCAACAATAGCCACCAATAAAG A	54
PSMP22 48	TCTGTTTGTGTTGGGTCAGGTCCTT C	CGAATACGTATGGAGAACTGCG CATC	58
PSMP22 55	CATCTAAACACAACCAATCTTGAA C	TGGCACTCTTAAATTGACGCAT	54
PSMP22 66	CAAGGATGGCTGAAGGGCTATG	TTTCCAGCCCACACCAGTAATC	58
PSMP22 67	GGAAGGCGTAGGGATCAATCTCA C	ATCCACCCGACGAAGGAAACGA	60
Xipes00 93	GGATCTGCAGGTTTGGACAT	CCAAGCACTGAAACATGCAC	57
Phil2275 62	TGATAAAGCTCAGCCACAAGG	ATCTCGGCTACGGCCAGA	56
Xcup14	TACATCACAGCAGGGACAGG	CTGGAAAGCCGAGCAGTATG	53
Xcup63	GTAAAGGGCAAGGCAACAAG	GCCCTACAAAATCTGCAAGC	53
XTXP27 8	GGG TTT CAA CTC TAG CCT ACC GAA CTT CCT	ATG CCT CAT CAT GGT TCG TTT TGC TT	50

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77 Data analysis

78 Microsatellite allele distribution data obtained from Genscan® software Version 4.1 were converted into suitable formats
79 for statistical analysis. Allelic size data for each SSR locus was used to estimate percentage of polymorphic loci,
80 Shannon's information index (I), Nei's gene diversity, observed (H_o) and expected (H_e) heterozygosities using Power
81 Marker version 3.25 (10). Cluster analysis was performed based on Nei's distance matrix using GenALEX6.2 (11).

82 RESULTS

83 The SSR markers used in this study generated 90 alleles in 23 Napier grass accessions. The number of alleles
84 detected for each primer pair ranged from 2 (CTM10, CTM59, PSMP2235, PGIRD25) to 13 (CTM8) with an average of
85 5.29 allele pair (Table 2). The frequency of the major alleles in each marker locus ranged from 0.23 locus (PSMP2267)
86 to 0.96 (CTM59). Polymorphic Information Content value for the assayed marker loci ranged from 0.04 (PGIRD25) to

0.85 (PSMP2267) with an average of 0.5. Similarly, marker locus PSMP2267 had the highest expected and observed heterozygosity of 0.86 and 0.95, respectively. In relation, marker locus PGIRD25 exhibited the least expected and observed heterozygosity all at 0.04 (Table 2).

Table 2. Genetic diversity parameters averaged across all groups and loci for 23 Napier grass clones

Primer	Major allele frequency	Number of alleles	Gene diversity/ expected heterozygosity (He)	Observed heterozygosity (Ho)	Polymorphic information content (PIC)
CTM10	0.52	2	0.49	0.87	0.37
CTM59	0.96	2	0.08	0.09	0.08
CTM8	0.29	13	0.82	0.89	0.80
CTM27	0.43	4	0.64	0.96	0.57
PGIRD21	0.37	9	0.80	0.52	0.78
PGIRD57	0.76	5	0.40	0.04	0.38
PSMP2248	0.63	4	0.53	0.22	0.47
Xipes0093	0.60	5	0.58	0.80	0.53
Phil227562	0.54	4	0.58	0.93	0.50
Xcup14	0.79	4	0.35	0.36	0.38
PSMP2266	0.47	5	0.66	1	0.61
PSMP2235	0.59	2	0.48	0.65	0.37
PGIRD25	0.98	2	0.04	0.04	0.04
PSMP2267	0.23	11	0.86	0.95	0.85
PSMP2255	0.33	7	0.76	0.78	0.72
XTXP278	0.64	6	0.53	0.72	0.47
xcup63	0.48	5	0.66	0.96	0.61
Mean	0.57	5.29	0.54	0.63	0.50

The proportion of rare alleles (private alleles) within the Napier grass accessions ranged from 0.00 in clones 41, 75, 76, 79, ANF, kakamega1 and RBN to 0.278 in clone 16814. The highest Shannon information index (0.539) was recorded in clone 16785 while the least was recorded in clone 16814 and 105. In relation, the highest expected heterozygosity (He) was recorded in clone 16785 while the least was recorded in clone 16814 and 105. Napier grass accession 16785 showed the highest number of effective alleles (Ne) while accession 105 had the least. Similarly, percentage polymorphic loci ranged from 27.8% (16814 and 105) to 77.8% in clone 16785 (Table 3).

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Table 3. Mean number of different loci (N_a), number of effective loci (N_e) Expected heterozygosity (H_e), Shannon index (I) and percentage polymorphism across the 23Napier grass clones

Population	N_e	I	Proportion of private Alleles	H_e	%Polymorphism
19	1.500	0.385	0.222	0.278	55.6
41	1.667	0.462	0.000	0.333	66.7
75	1.444	0.347	0.000	0.250	50.0
76	1.667	0.462	0.000	0.333	66.7
79	1.556	0.385	0.000	0.278	55.6
97	1.389	0.347	0.111	0.250	50.0
103	1.556	0.424	0.056	0.306	61.1
104	1.611	0.424	0.111	0.306	61.1
105	1.000	0.193	0.056	0.139	27.8
112	1.278	0.308	0.111	0.222	44.4
117	1.556	0.462	0.056	0.333	66.7
16702	1.222	0.308	0.056	0.222	44.4
16785	1.778	0.539	0.111	0.389	77.8
16789	1.500	0.385	0.056	0.278	55.6
16805	1.556	0.385	0.111	0.278	55.6
16814	1.222	0.193	0.278	0.139	27.8
16815	1.556	0.424	0.056	0.306	61.1
79SN	1.444	0.347	0.056	0.250	50.0
ANF	1.333	0.308	0.000	0.222	44.4
kakamega1	1.500	0.385	0.000	0.278	55.6
kakamega2	1.556	0.385	0.056	0.278	55.6
kakamega3	1.556	0.385	0.056	0.278	55.6
RBN	1.222	0.308	0.000	0.222	44.4

Pairwise comparison of genetic distance revealed big difference among the Napier grass clones ranging from 0.11 (between kakamega1 and kakamega2 and kakamega2 and kakamega 3) to 1.099 between clone 16814 and 105 (Table 4). Principal Coordinate Analysis was calculated from dissimilarity coefficients for two first axes coordinates with positive eigen values. The axes accounted for 52.8% variation with first axis accounting for 29.8% while the second axis accounted for 23.0%. Principal Coordinate Analysis did not group the clones into clear structures. However, clones kakamega1, kakamega2, kakamega3 and 16805 and clone 112 and ANF also grouped together, respectively (figure 1).

110 The UPGMA dendrogram based on pairwise Nei's genetic distance showered two major clusters; one consisting of only
111 clone 16814 and the other consisting of the rest of the clones (figure 2).
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Table 4. Nei's Unbiased genetic distance of the 23 Napier grass clones based on SSR analysis

[illegible]

64	1 2 3	3 5	2 7 3	14 9	31 8	17 9	36 7	69 3	34 2	24 1	58 8												78 5
0.6 52	0. 2 9 2	0. 5 6 3	0. 4 8 6	0. 26	0. 20 7	0. 44 4	0. 38	0. 68 7	0. 42 2	0. 35 3	0. 7	0. 23 3	0										16 78 9
0.6 52	0. 2 3 5	0. 4 6 2	0. 4 5 1	0. 15 8	0. 28 5	0. 38	0. 55	0. 80 5	0. 45 3	0. 29 2	0. 78 3	0. 17 6	0. 23 4	0									16 80 5
0.6 15	0. 5 6 3	0. 4 2 4	0. 4 9 6	0. 42 7	0. 54 1	0. 73 8	0. 91 3	1. 09 9	0. 93 9	0. 67 2	0. 93 9	0. 69 3	0. 68 7	0. 68 7	0								16 81 4
0.5 5	0. 2 0 9	0. 4 6 9	0. 3 5 9	0. 15 7	0. 31 5	0. 29 4	0. 41 9	0. 82 2	0. 63 5	0. 32 7	0. 75 8	0. 17 9	0. 31 9	0. 29	0. 69 9	0							16 81 5
0.6 37	0. 2 8 8	0. 4 2 3	0. 3 7	0. 25 8	0. 33 6	0. 28 6	0. 53 8	0. 78 8	0. 44 7	0. 37 7	0. 80 8	0. 20 1	0. 23 2	0. 28 5	0. 64 4	0							79 S N
0.8 28	0. 4 6 5	0. 6 8 5	0. 7 3 1	0. 42 2	0. 41 7	0. 63 5	0. 45 9	0. 69 8	0. 47	0. 53 4	0. 59 9	0. 34 2	0. 23	0. 39 2	0. 73 5	0. 52 6	0. 38 8	0					A N F
0.5 04	0. 1 3	0. 4 6 2	0. 4 5 1	0. 15 8	0. 25 8	0. 34 9	0. 47 8	0. 72 5	0. 45 3	0. 38 5	0. 66 1	0. 14 9	0. 18 2	0. 15 8	0. 58 2	0. 23 5	0. 23 2	0. 33 5	0				ka ka 1
0.4 7	0. 2 3 5	0. 3 9 9	0. 3 5 3	0. 23 4	0. 28 5	0. 26 2	0. 44 4	0. 76 4	0. 45 3	0. 38 5	0. 74 1	0. 12 2	0. 15 8	0. 23 4	0. 65 1	0. 29	0. 23 2	0. 33 5	0. 11	0			ka ka 2
0.5 75	0. 2 3 5	0. 4 3	0. 4 1 7	0. 15 8	0. 25 8	0. 31 9	0. 31 9	0. 72 5	0. 45 3	0. 35 3	0. 78 3	0. 17 6	0. 15 8	0. 20 8	0. 58 2	0. 18 2	0. 23 2	0. 28 1	0. 13 4	0. 11	0		ka ka 3
0.7	0. 4 6 5	0. 6 1 1	0. 5 3 4	0. 36 4	0. 33 2	0. 59 7	0. 56 1	0. 62 9	0. 70 8	0. 46 5	0. 63 4	0. 47 5	0. 30 8	0. 42 2	0. 69 8	0. 45 9	0. 50 9	0. 53 3	0. 39 2	0. 42 2	0. 42 2	0	R B N

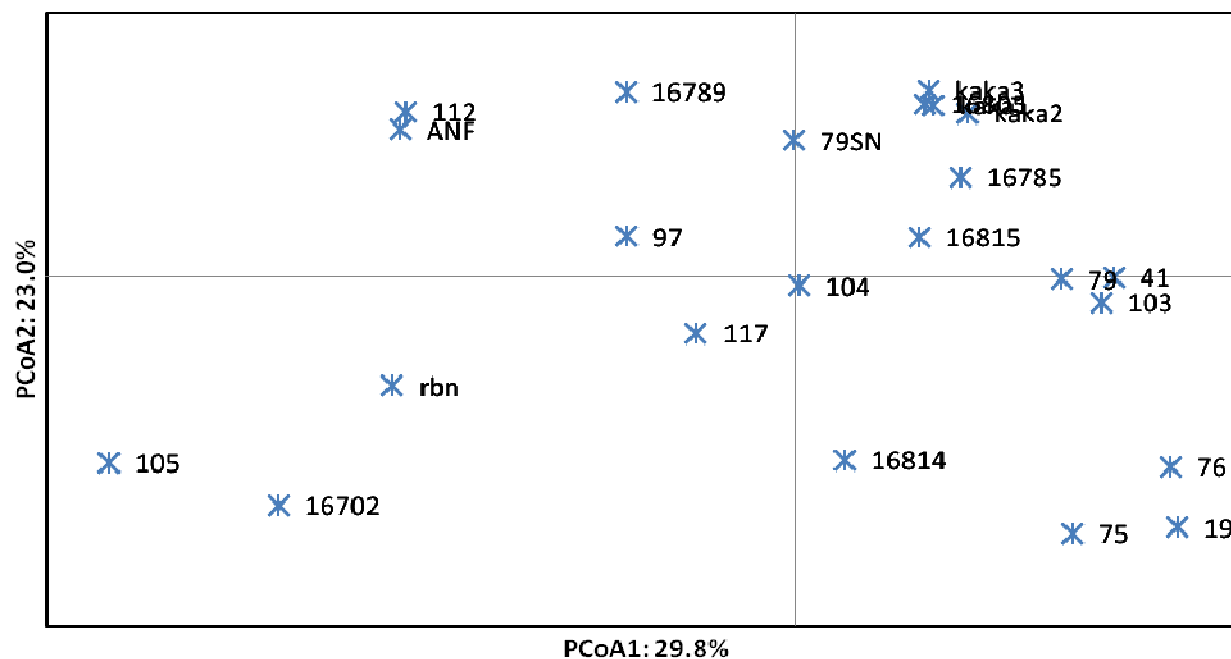


Figure 1: PCoA scatter plot showing the clustering of the 23 Napier grass clones

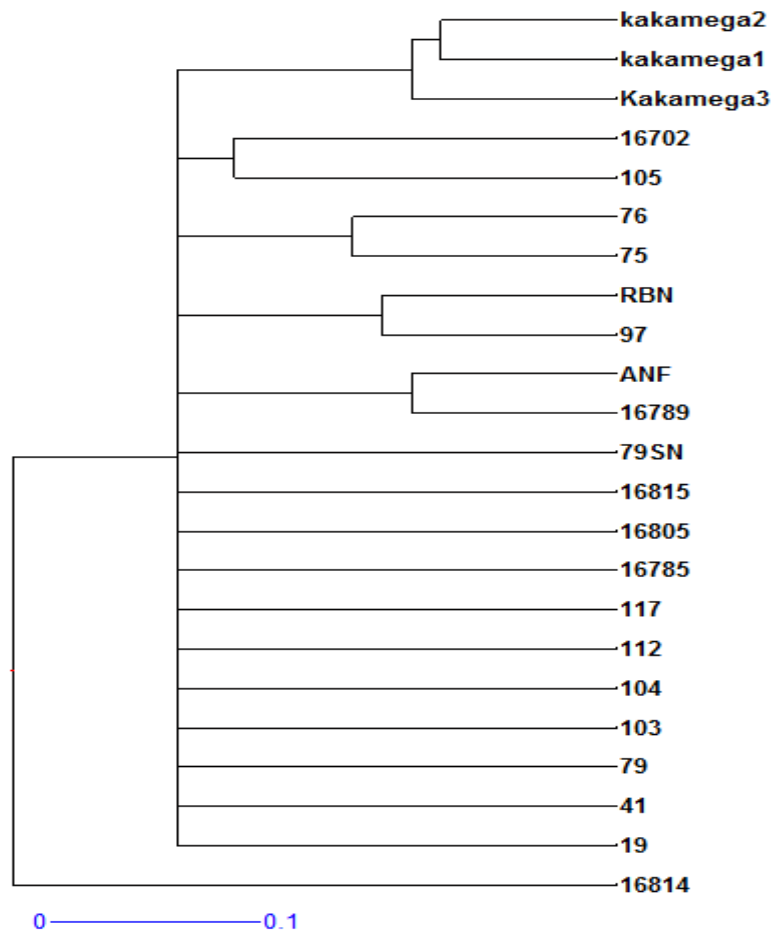


Figure 2. UPGMA Neighbour Joining dendrogram of 23 Napier grass clones computed from 17 SSR markers using Darwin hierarchical clustering

DISCUSSION

Genetic characterization of cultivars is an important step in any breeding programs for selection of appropriate parental lines (12). Simple Sequence Repeat markers have been reported as the most adequate in detecting relationships among closely related materials as well as obtaining specific genetic fingerprints (13). In this study, the 17 SSR markers used produced high mean polymorphic information content suggesting that they are highly informative and able to discriminate among the different clones. The variation in polymorphic information content values is influenced by the type of markers used (14).

This study revealed high genetic variation among the clones evaluated, majority of which had private alleles. Similarly, high variation in genetic distance between clones was revealed, with the highest genetic distance between clones 16814 and 105. This was further supported by the dendrogram which grouped clone 16814 different from the rest. The grouping of clone 16814 in a cluster different from that of clone 16815 and 16805, yet all originate from United States of America (15) shows that the clustering was not based on the origin of the clones. This is contrary to the findings of Lowe *et al.* (2003 (1), but in agreement with Wanjala *et al.* (2013 (16). The clustering together of the other clones most of which originate in Africa is a proof that Africa is the center of domestication (8), as such it houses majority of the pennisetum gene pools (17). The poor clustering of accessions is possibly due to absence or low gene flow since Napier grass is clonally propagated. However, the genetic closeness of Kakamega 1, Kakamega 2 and Kakamega 3 indicates that they share almost all alleles and were collected from the same area - Kakamega in Kenya.

CONCLUSION

Based on the foregoing, therefore, clones evaluated in this study are diverse with unique genetic traits that can be exploited by breeding programs to produce Napier grass clones with farmer preferred traits. Clone 16814, which is the most distant of all, is better suited for improvement of the rest. Clones Kakamega 1, Kakamega 2, and Kakamega 3, due to their high level of relatedness, only one can be selected for genetic improvement with other clones.

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