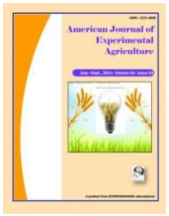

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Genetic Characterization of Alupe Napier Grass Accessions Based on Simple Sequence Repeat Markers

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Authors' contributions

This work was carried out in collaboration between all authors. Author GK designed field study, conducted laboratory and data analysis, participated in the writing and revision of the manuscript. Author JK sourced the Napier grass clones, participated in design and planting field experiment and revision of the manuscript. Author CN participated in planting field experiment, collected leaf samples and participated in revision of the manuscript. All authors read and approved the final manuscript.

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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. All markers were polymorphic with the most discriminative being PSMP2267. Average gene diversity among the Napier grass clones was 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. Principal Co-ordinates Analysis did not group the clones in a definitive structure, with most clones scattered. However, the SSR

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markers grouped the clones into two clusters derived from a dendogram generated by Neighbour joining analysis with clone 16814 being alone in a cluster. Clone Kakamega1, Kakamega2 and Kakamega3 as very closely related. These findings are key in guiding selection of clones for inclusion in breeding programs and conservation of Napier grass.

Keywords: Genetic diversity; genetic relationships; Pennisetum pupureum; population structure-simple sequence repeats transferability.

1. 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]. The grass can also withstand repeated cutting with 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 the 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 to these production constraints. This requires a well characterized and inventoried germplasm; which is lacking in the case of Napier grass in East African countries including 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, in Ethiopia whose genetic diversity is not known.

Various methods for estimating diversity in a plant population exist and use of simple sequence repeats (SSRs) has become 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. Besides, Napier grass is a tetraploid (2n = 4x = 28) with triploid and hexaploid hybrids occurring between it and pearl millet [8]. This makes establishing microsatellites that adequately discriminate the different ploidy levels difficult. The available option is through cross-amplification using SSR markers of closely related species [9]. This study, therefore, determined the genetic variability among Napier grass clones maintained at Kenya Agricultural Research Institute – Alupe at station through cross- amplification using SSR markers of closely related organisms.

2. MATERIALS AND METHODS

2.1 Sample Collection and Analysis

Twenty two Napier grass clones (19, 41, 75, 76, 79, 97, 103, 104, 105, 112, 117, 16702, 16789, 16805, 16814, 16815, 79SN, ANF, Kakamega1, Kakamega2, kakamega3 and RBN) 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 and silica gels and packed in a box. These were transferred to Bioscience Eastern and Central Africa at International Livestock Research Institute (BecA-ILRI) - Nairobi for genotyping.

In the laboratory, 1.5 g of a leaf was extracted from each leaf sample and ground in mortar in liquid Nitrogen. Total plant DNA was extracted using cetyltrimethylammonium bromide (CTAB) method [10] and diluted to 100 µ using double distilled water. The DNA concentration was determined using Nanodrop UV spectrometry at A260 and A280 while the integrity of DNA was tested on 1.2% agarose gel electrophoresis in TBE buffer stained with gel red. From these,

template DNA was made from an aliquot in a 1.5 ml tube and diluted to 50 ng/µ.

2.2 DNA Amplification with Microsatellite Markers

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

2.3 Data Analysis

Microsatellite allele distribution data obtained from Genscan®software Version 4.1 were converted into suitable formats for statistical analysis. Allelic size data for each SSR locus was used to estimate percentage of polymorphic loci, Shannon's information index (*I*), Nei's gene diversity, observed (H_o) and expected (H_e) heterozygosities using Power Marker version 3.25 [11]. Cluster analysis was performed based on Nei's distance matrix using GenALEX6.2 [12].

3. RESULTS

The SSR markers used in this study generated 90 alleles in 23 Napier grass accessions. The number of alleles detected for each primer pair ranged from 2 (CTM10, CTM59, PSMP2235, PGIRD25) to 13 (CTM8) with average of 5.29 (Table 2). The frequency of the major alleles in each marker locus ranged from 0.23 (PSMP2267) to 0.98 (PGIRD25). Polymorphic Information Content for the assayed marker loci ranged from 0.04 (PGIRD25) to 0.85 (PSMP2267) with average of 0.5. The observed and expected heterozygosity generated by the markers was moderate. Marker PSMP2267 had the highest expected and observed heterozygosity of 0.86 and 0.95, respectively while PGIRD25 exhibited the least expected and observed heterozygosity both at 0.04 (Table 2).

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

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 1.00 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% total 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 grouped together, respectively (Fig. 1). The UPGMA dendogram based on pairwise Nei's genetic distance showed two major clusters; one consisting of only clone 16814 and the other consisting of the rest of the clones (Fig. 2).

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 (^{o}C)
CTM-10	GAGGCAAAAGTGGAAGACAG	TTGATTCCCGGTTCTATCGA	52
CTM-27	GTTGCAAGCAGGAGTAGATCGA	CGCTCTGTAGGTTGAACTCCTT	52
CTM-59	TCCTCGACATCCTCCA	GACACCTCGTAGCACTCC	54
CTM-8	GCTGCATCGGAGATAGGGAA	CTCAGCAAGCACGCTGCTCT	52
PGIRD21	GCTATTGCCACTGCTTCACA	CCACCATGCAACAGCAATAA	54
PGIRD25	CGGAGCTCCTATCATTCCAA	GCAAGCCACAAGCCTATCTC	58
PGIRD57	GGCCCCAAGTAACTTCCCTA	TCAAGCTAGGGCCAATGTCT	56
PSMP2235	GCTTTTCTGCTTCTCCGTAGAC	CCCAACAATAGCCACCAATAAAGA	54
PSMP2248	TCTGTTTGTTTGGGTCAGGTCCTTC	CGAATACGTATGGAGAACTGCGCATC	58
PSMP2255	CATCTAAACACAACCAATCTTGAAC	TGGCACTCTTAAATTGACGCAT	54
PSMP2266	CAAGGATGGCTGAAGGGCTATG	TTTCCAGCCCACACCAGTAATC	58
PSMP2267	GGAAGGCGTAGGGATCAATCTCAC	ATCCACCCGACGAAGGAAACGA	60
Xipes0093	GGATCTGCAGGTTTGGACAT	CCAAGCACTGAAACATGCAC	57
Phil227562	TGATAAAGCTCAGCCACAAGG	ATCTCGGCTACGGCCAGA	56
Xcup14	TACATCACAGCAGGGACAGG	CTGGAAAGCCGAGCAGTATG	53
Xcup63	GTAAAGGGCAAGGCAACAAG	GCCCTACAAAATCTGCAAGC	53
XTXP278	GGG TTT CAA CTC TAG CCT ACC GAA CTT CCT	ATG CCT CAT CAT GGT TCG TTT TGC TT	50

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

Table 3. Mean number of effective loci (*ne*), shannon index (*i*), proportion of private alleles, expected heterozygosity (*he*) and percentage polymorphism across the 23 Napier grass clones

Population	Ne	I	Proportion of private alleles	He	% 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

Table 4. Nei's unbiased genetic distance of the 23 Napier grass clones based on SSR analysis

19	41	75	76	79	97	103	104	105	112	117	16702	16785	16789	16805	16814	16815	79SN	ANF	kaka1	kaka2	kaka3	RBN	
0																							19
0.263	0																						41
0.312	0.259	0																					75
0.208	0.239	0.179	0																				76
0.345	0.105	0.207	0.181	0																			79
0.6	0.288	0.519	0.442	0.207	0																		97
0.412	0.155	0.315	0.238	0.262	0.373	0																	103
0.587	0.426	0.612	0.426	0.38	0.503	0.562	0																104
0.938	0.839	0.872	0.795	0.725	0.749	0.913	0.699	0															105
0.974	0.57	0.852	0.822	0.552	0.576	0.635	0.674	0.698	0														112
0.683	0.365	0.511	0.434	0.292	0.377	0.359	0.535	0.634	0.57	0													117
0.741	0.608	0.647	0.775	0.587	0.685	0.897	0.597	0.852	0.922	0.87	0												16702
0.464	0.123	0.35	0.273	0.149	0.318	0.179	0.367	0.693	0.342	0.241	0.588	0											16785
0.652	0.292	0.563	0.486	0.26	0.207	0.444	0.38	0.687	0.422	0.353	0.7	0.233	0										16789
0.652	0.235	0.462	0.451	0.158	0.285	0.38	0.55	0.805	0.453	0.292	0.783	0.176	0.234	0									16805
0.615	0.563	0.424	0.496	0.427	0.541	0.738	0.913	1.000	0.939	0.672	0.939	0.693	0.687	0.687	0								16814
0.55	0.209	0.469	0.359	0.157	0.315	0.294	0.419	0.822	0.635	0.327	0.758	0.179	0.319	0.29	0.699	0							16815
0.637	0.288	0.423	0.377	0.258	0.336	0.286	0.538	0.788	0.447	0.377	0.808	0.201	0.232	0.285	0.64	0.344	0						79SN
0.828	0.465	0.685	0.731	0.422	0.417	0.635	0.459	0.698	0.47	0.534	0.599	0.342	0.23	0.392	0.735	0.526	0.388	0					ANF
0.504	0.13	0.462	0.451	0.158	0.258	0.349	0.478	0.725	0.453	0.385	0.661	0.149	0.182	0.158	0.582	0.235	0.232	0.335	0				kaka1
0.47	0.235	0.399	0.353	0.234	0.285	0.262	0.444	0.764	0.453	0.385	0.741	0.122	0.158	0.234	0.651	0.29	0.232	0.335	0.11	0			kaka2
0.575	0.235	0.43	0.417	0.158	0.258	0.319	0.319	0.725	0.453	0.353	0.783	0.176	0.158	0.208	0.582	0.182	0.232	0.281	0.134	0.11	0		kaka3
0.7	0.465	0.611	0.534	0.364	0.332	0.597	0.561	0.629	0.708	0.465	0.634	0.475	0.308	0.422	0.698	0.459	0.509	0.533	0.392	0.422	0.422	0	RBN

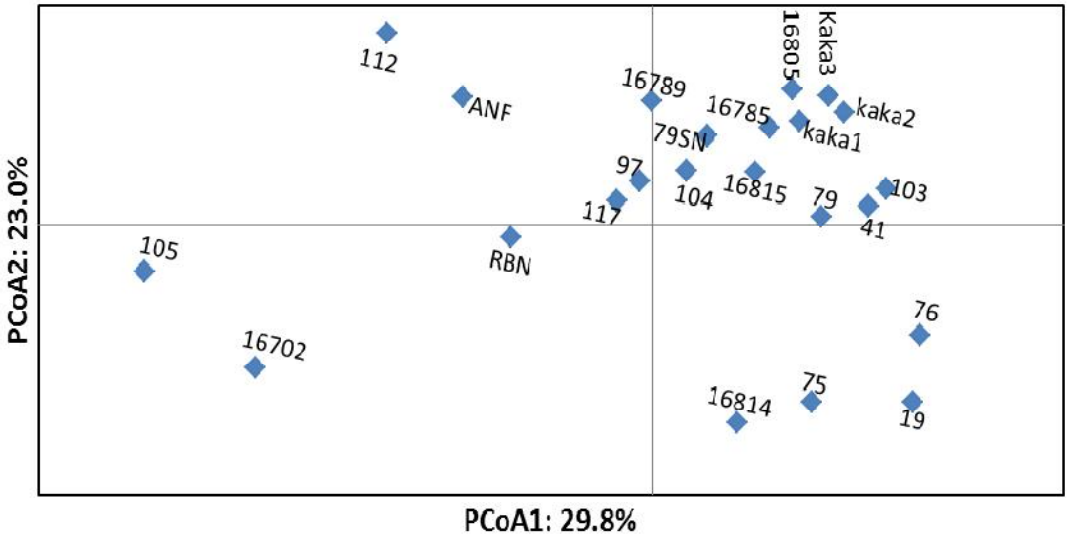

Fig. 1. PCoA scatter plot showing the clustering of the 23 Napier grass clones
Kaka = Kakamega

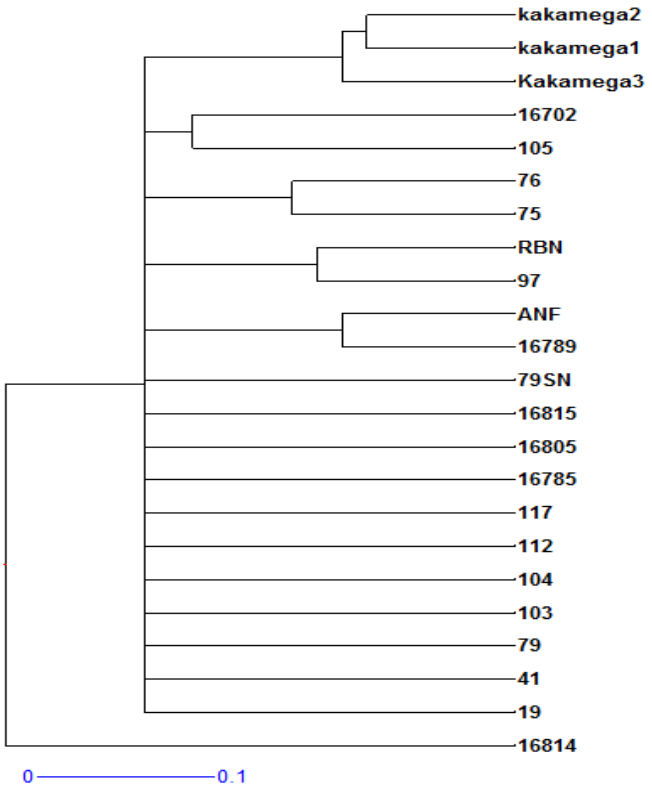

Fig. 2. UPGMA neighbour joining dendogram of 23 napier grass clones computed from 17 ssr markers using darwin hierachial clustering

4. DISCUSSION

Genetic characterization of cultivars is an important step in any breeding programs for selection of appropriate parental lines [13]. Several DNA marker systems for germplasm genetic characterization are available and SSRs have been found most adequate in detecting relationships among closely related materials as well as obtaining specific genetic fingerprints [14]. In this study, 17 SSR markers used produced high mean polymorphic information content suggesting that they are highly informative and able to discriminate among the different clones. According to Elibariki et al. [15] the ability to discriminate, however, varies from one marker to another, thus the most polymorphic marker was CTM8 while the least polymorphic was PGIRD25.

Both gene diversity and observed heterozygosity averaged across all loci was moderate. This result is in agreement with the findings of Wanjala et al. [16] who while working on Napier grass from east Africa region using AFLPs found moderate diversity among accessions. According to Bhandari et al. [17], Napier grass is of free pollination and high genetic diversity is expected from its natural crossings. The moderate genetic diversity revealed in this study is due to the fact that Napier grass grown onfarm is predominantly propagated by cuttings and subjected to high selection intensity by farmers. The markers revealed high number of private alleles in majority of the Napier grass clones. These, if included in breeding programs increase the chances of getting clones with farmer preferred traits.

The genetic distance revealed between the clones was generally high, with the highest distance being between clones 16814 and 105. This was further supported by the dendogram in which clone 16814 clustered different from the rest. This provides a basis for developing heterotic pool [18] from which crosses between genetically diverse parents can be made to produce progenies with higher genetic variation than those produced by closely related parents. The grouping of clone 16814 different from clone 16815 and 16805, yet all originate from United States of America [19] shows that the clustering was not based on the origin of the clones. This view contradicts the findings of Lowe et al. [1] who while using RAPDS reported that Napier grass accessions cluster corresponding to geographical location. However, it is in agreement with Wanjala et al. [16] who while using AFLPs reported that Napier grass did not cluster depending on their origin. The clustering together of the other clones most of which originate in Africa is a proof that Africa is the center of diversity [9], as such it houses majority of the *pennisetum* gene pools [8]. The loose clustering of accessions as revealed by PCA is possibly due to absence or low gene flow since Napier grass is clonally propagated. The genetic closeness of Kakamega 1, Kakamega 2 and Kakamega 3 indicates that they share most alleles and were collected from the same area known as Kakamega in Kenya.

5. CONCLUSION

Based on the foregoing, clones evaluated in this study are diverse with multitudes of private alleles which if found useful can be exploited in breeding to improve Napier grass. As such, Clone 16814, which is the most distant to all, is better suited for improvement of the rest of the clones if its attributes are found superior to those in others. Clones Kakamega1, Kakamega2, and Kakamega3 are more less the same, hence if any genetic improvement is to be carried out; it has to be with other distant clones.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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