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CHARACTERIZATION OF ELITE MAIZE INBRED LINES FOR DROUGHT TOLERANCE USING SIMPLE SEQUENCE REPEATS MARKERS

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ABSTRACT

The development of drought tolerant maize has been limited by the suggested complexity of the environment on drought phenotypic traits. However, some simple sequence repeats (SSRs) molecular markers linked to drought tolerance via quantitative trait loci (QTL) have been identified in maize but their use requires validation on newly developed elite maize inbred lines. This study therefore aims to validate 19 selected SSR markers linked to maize drought tolerance and determine the genetic diversity of sixty-eight elite maize inbred lines. Genomic DNA was extracted with a CTAB method and the PCR products were separated on agarose gel with auto radiograms visually scored for polymorphic bands to establish a data matrix. Assessment of the genetic links among the inbred lines was carried out using cluster analysis. The 68 maize inbred lines were clustered based on a matrix of genetic similarity Jaccard using the UPGMA algorithm. Some of the markers that were informative included P-bnlg238, Phi037, P-bnlg1179 and Umc2214 and these showed significant group differentiation among the inbred lines. Marker Umc1447, Umc1432 and Umc2359 were among the markers with monomorphic bands, while Phi034, Bnlg1074 and P-umc1542 showed no characterized bands. The polymorphism information content (PIC) value of the informative markers ranged from 0.13 (Bnlg434) to 0.76 (P-bnlg238). The cluster analysis classified the maize inbred lines into four groups based on the

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SSR data. The exploitation of information of genetic diversity among the inbred maize lines to develop drought tolerant hybrids is hereby discussed.

Keywords: Drought tolerance, maize, marker validation, Simple Sequence Repeat markers

1. INTRODUCTION

Maize (*Zea mays* L.) ranks third in the world after wheat and rice with respect to production area and yield (Mohammedreza, 2011). A large amount of land has been allocated for its cultivation but its actual yields relative to potential yields are limited by abiotic and biotic stresses. Developing varieties tolerant to environmental stress, in particular drought, has the potential to increase production. Due to the complexity of crop drought tolerance traits, previous achievements have not been sustained due to insufficient genetic variation to select for drought tolerance, the complex interaction between drought and other environmental stresses (e.g. heat) and the use of inappropriate accession selection techniques (Shiri *et al.*, 2010a, b). Despite the complex genetical, biochemical, physiological and molecular traits linked to drought tolerance, crop improvement through the use of marker assisted selection (MAS) has had its successes. For example, Mohammedzri, (2011) identified markers Umc2359, Umc1432, Umc1862 and Umc1719 that are related to yield under drought stress in maize which could be further validated and potentially deployed in molecular marker-assisted (MAS) breeding for drought tolerance. Developments such as these in molecular genetics have provided plant breeders with the tools to identify and select Mendelian inheritance principles (principles of segregation and independent assortment of genes) underlying complex stress tolerance, genetic and environmental traits (Ribaut and Ragot, 2007). The advent of abundant DNA-based molecular markers has allowed the construction of genetic maps (Helentjaris *et al.*, 1986, Rahman *et al*., 2011). Most of the research efforts have been directed towards the development of MAS to determine genetic diversity in maize (Phelps *et al.*, 1996, Taramino and Tingay 1996, Pinto *et al.*, 2003; Park *et al*., 2009; Pandey *et al.*, 2016). Microsatellite markers are among the DNA markers that show genetic differences between genotypes due to their high polymorphism, and can hence, be used for maize genetic mapping and evaluating germplasm diversity (Jonah *et al.*, 2011). Microsatellites or simple sequence repeats (SSRs) are repeating sequences of 2-6 bp segments of 20 to 100 bp (Goldstein and Schlotterer, 1998; Agrawal *et al*., 1999), 2-8 bp repeats (Armour *et al.*, 1999), 1-6 bp repeats (Goldstein and Pollock, 1997), 1-5 bp repeats (Schlotterer, 1998). They have several features that distinguish them from other genetic markers; which include their ease of application as a PCR based marker and their cheaper identification using an agarose gel system (Bantte and Prasanna, 2003, Mohammedzra, 2011); their co-dominance and detection of heterozygote from homozygote; their frequency in the genome and their high level of allele variation; they also have high rates of mutation on average of 5×10^{-4} mutation per locus, per

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generation (Pandey *et al*., 2016). This results in high levels of allelic diversity in the microsatellite locus (Ovesna *et al*., 2002). The high level of microsatellite markers in maize (Senior e*t al*., 1998) suggests they can be exploited for identifying genetic diversity as well as evolutionary studies (Matsuoka *et al*., 2002). It is however not surprising that the genetic diversity in *Zea mays* was lower compared with its wild relatives e.g. *Zea diploperennis, Zea luxurians,* most likely due to self-fertilization and human improvement programmes (Mohammedzra, 2011).

Among the sequences of 2 and 3 nucleotides used as microsatellite, the 3 nucleotides ones have the highest frequency in the maize genome (Chin *et al*., 1996). Microsatellites with longer repeats are less common and data regarding their evolution is limited (Li *et al.*, 2002). It has also been reported that the most frequent polymorphism were found in the repetition of 2 and 3 nucleotides (Kantety *et al.*, 1995). Reduced diversity due to crop development is particularly apparent when looking for adaptation to biotic and abiotic stresses (Matsuoka *et al.*, 2002). There are, however, some SSR markers which are linked to maize drought tolerance, but these have not been validated with the elite modern drought tolerant inbred lines, such as those found at the Cereal Research Program, NaCRRI, Uganda (Sserumaga *et al*., 2014). This study therefore aims to validate selected maize SSR markers linked to drought tolerance with elite drought tolerant maize inbred lines and determine the genetic diversity among the elite inbred lines of the NaCRRI Program.

2. MATERIALS AND METHODS

Study Location and Plant Material

This study was carried out at the Biotechnology Laboratory of the Makerere University of Agricultural Research Institute, Kabanyolo (MUARIK), Uganda. Sixty-eight drought tolerance maize inbred lines were selected from the maize breeding program at the National Crop Resource Research Institute (NaCRRI), Namulonge, Uganda for this study (Table 1).

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Table 1: Pedigree and origin of maize inbred lines characterised for drought tolerance and three drought susceptible lines (checks)

S/N	Name	Pedigree	Characteristics	Origin
1	CKLMARS1F60677	([Ent320:92SEW2-77/[DMRESR-W]EarlySel-#I-2-4-B/CML386]-B-11-3-B-2-#-B*4/[INTA-2-1-3/INTA-60-1-2]-X-11-6-3-BBB)F2-101-6-1-B-B-B	Drought Tolerant	CIMMYT
2	DL111901	DL111901-B	Drought Tolerant	CIMMYT
3	CKDHL120671	CKDHL120671-B	Drought Tolerant	CIMMYT
4	CML488	CML488-B	Drought Tolerant	CIMMYT
5	CKDHL0221	CKDHL0221-B	Drought Tolerant	CIMMYT
6	CML537	CML537-B	Drought Tolerant	CIMMYT
7	CKL08018	CKL08018-B	Drought Tolerant	CIMMYT
8	CKDHL120312	CKDHL120312-B	Drought Tolerant	CIMMYT
9	CML545	CML545-B	Drought Tolerant	CIMMYT
10	CML445	CML445-B	Drought Tolerant	CIMMYT
11	CKLTI0200	(CML495*/OFP14)-8-2-6-1-2-B-B-B-B	Drought Tolerant	CIMMYT
12	CKDHL0214	CKDHL0214-B	Drought Tolerant	CIMMYT
13	CKL14154	CKL14154-B	Drought Tolerant	CIMMYT
14	CKDHL0323	CKDHL0323-B	Drought Tolerant	CIMMYT
15	CML489	CML489-B	Drought Tolerant	CIMMYT
16	CML494	CML494-B	Drought Tolerant	CIMMYT
17	CML312	CML312-B	Drought Tolerant	CIMMYT
18	CKLTI0136	(CML494*/OFP9)-12-2-1-1-1-B-B-B-B	Drought Tolerant	CIMMYT
19	CKDHL120918	CKDHL120918-B	Drought Tolerant	CIMMYT
20	CKLTI0272	(CML537*/OFP106)-5-2-2-3-2-B-B-B-B	Drought Tolerant	CIMMYT
21	CML322	CML322-B	Drought Tolerant	CIMMYT
22	CKL12136	CKL12136-B	Drought Tolerant	CIMMYT
23	CML444	CML444-B	Drought Tolerant	CIMMYT
24	CML548	CML548-B	Drought Tolerant	CIMMYT
25	CZL03011	CZL03011-B	Drought Tolerant	CIMMYT
26	CKDHL0295	CKDHL0295-B	Drought Tolerant	CIMMYT
27	CML445*2/S3512ZF 2-4-1-1	CML445*2/S3512ZF2-4-1-1-B	Drought Tolerant	CIMMYT
28	CML247-IR	CML247-IR	Drought Tolerant	CIMMYT
29	CML539	CML539-B	Drought Tolerant	CIMMYT
30	CKL08023	CKL08023-B	Drought Tolerant	CIMMYT
31	P100C6-200-1-1-#-#-B-B-B-B-B	P100C6-200-1-1-#-#-B-B-B-B-B-B	Drought Tolerant	CIMMYT

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32	CML550	CML550-B	Drought Tolerant	CIMMYT
33	CML247	CML247-B	Drought Tolerant	CIMMYT
34	CZL096	CZL096-B	Drought Tolerant	CIMMYT
35	DL111904	DL111904-B	Drought Tolerant	CIMMYT
36	La Posta Seq C7-F64-2-6-2-2-B-B-B-B	La Posta Seq C7-F64-2-6-2-2-B-B-B-B-B	Drought Tolerant	CIMMYT
37	CML312-IR	CML312-IR	Drought Tolerant	CIMMYT
38	CML78	CML78-B	Drought Tolerant	CIMMYT
39	CML443	CML443-B	Drought Tolerant	CIMMYT
40	CKDHL0500	CKDHL0500-B	Drought Tolerant	CIMMYT
41	CML 543	CML543-B	Drought Tolerant	CIMMYT
42	CML547	CML547-B	Drought Tolerant	CIMMYT
43	CKDHL0089	CKDHL0089-B	Drought Tolerant	CIMMYT
44	CKDHL0109	CKDHL0109-B	Drought Tolerant	CIMMYT
45	CKDHL0159	CKDHL0159-B	Drought Tolerant	CIMMYT
46	CKDHL0165	CKDHL0165-B	Drought Tolerant	CIMMYT
47	CKDHL0214	CKDHL0214-B	Drought Tolerant	CIMMYT
48	CKDHL0227	CKDHL0227-B	Drought Tolerant	CIMMYT
49	CKDHL0228	CKDHL0228-B	Drought Tolerant	CIMMYT
50	CKDHL0231	CKDHL0231-B	Drought Tolerant	CIMMYT
51	CKDHL0250	CKDHL0250-B	Drought Tolerant	CIMMYT
52	CKDHL0282	CKDHL0282-B	Drought Tolerant	CIMMYT
53	CKL05007	CKL05007-B	Drought Tolerant	CIMMYT
54	CKL05017	CKL05017-B	Drought Tolerant	CIMMYT
55	CKL05018	CKL05018-B	Drought Tolerant	CIMMYT
56	CKL05019	CKL05019-B	Drought Tolerant	CIMMYT
57	CKL05022	CKL05022-B	Drought Tolerant	CIMMYT
58	CML202	CML202-B	Drought Tolerant	CIMMYT
59	CML312	CML312-B	Drought Tolerant	CIMMYT
60	CML395	CML395-B	Drought Tolerant	CIMMYT
61	CML442	CML442-B	Drought Tolerant	CIMMYT
62	CML444	CML444-B	Drought Tolerant	CIMMYT
63	CML536	CML536-B	Drought Tolerant	CIMMYT
64	CML539	CML539-B	Drought Tolerant	CIMMYT
65	DTPYC9-F46-1-2-1-2-B-B-B-B	DTPYC9-F46-1-2-1-2-B-B-B-B	Drought Tolerant	CIMMYT
66	NML 85	NML85	Drought Susceptible	NACRRI -2015
67	NML 88	NML88	Drought Susceptible	NACRRI -2015
68	NML 97	NML97	Drought Susceptible	NACRRI -2015

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DNA Extraction

Total genomic DNA of each maize inbred line was extracted from two to three growing leaves collected from the maize plants at the 4 to 5 leaf stage. Five hundred mg of leaf tissue was ground with mortar in 1000 µl CTAB buffer (CTAB 2 g, EDTA (0.5 M, pH 8.0) 10 mls, Tris base (1 M, pH 8.0) 10 mls, PVP 40 2 g, $NaSO_4$ 1 g, NaCl 8.18 g, 1 ml BME), and then transferred to Eppendorf tube and incubated for 15 minutes while gently inverting the tube every five minutes to ensure uniform heat distribution. The samples were then centrifuged at 13,000 rpm for 5 minutes after which 700 µl were pipetted into a freshly labelled tube. Seven hundred µl of chloroform:Iso-amyl alcohol (24:1) was added to achieve a ratio of sample to chloroform:Iso-amyl alcohol of 1:1. This mixture was vortexed for five minutes and centrifuged at 13,000 rpm for 10 minutes. The aqueous layer was then pipetted off. Equal volumes of chilled isopropanol was then added and incubated at -20ºC overnight. The incubated samples were then centrifuged to separate the pellet, after which the isopropanol was poured off and the pellet washed with 80% EtOH. The ethanol was poured off and the pellet air dried, after which the nucleic acid was reconstituted in nuclease free water (Saghai-Maroof *et al*., 1984; Doyle and Doyle 1990; Padilla-Ramirez *et al*., 2002). The quantity and quality of the extracted DNA were evaluated with a NanoDrop Spectrophotometer (BIONEER).

PCR Amplification Conditions and Gel Electrophoresis

Nineteen Simple Sequence Repeats (SSRs) primers (Table 2) were chosen based on repeat unit and bin location to provide uniform coverage of the entire maize genome from the Maize GDB database (Maize Genetics and Genomics Database, 2016). These SSR markers were used for Polymerase Chain Reaction (PCR). A total PCR reaction volume of 10 µl was used containing 5 µl Premix (AccuPower® PCR Master Mix), 0.25 µl forward primer, 0.25 µl reverse primer, (BIONEEER), 3.5 µl Autoclave water and 1 µl DNA template. An ARKTIK 96 well thermal cycler was used for the PCR amplification of the DNA with the following temperature profiles: 1 initial denaturation step at 95°C for 5 min followed by 35 cycles at 94ºC for 30 sec, AºC for 40 sec and 72ºC for 40 sec and a final extension cycle at 72ºC for 5 min. AºC refers to the appropriate annealing temperature of the primers as presented in Table 2. Amplification reaction products were separated on a 3% (w/v) superfine agarose gels (Metaphor) in 1X TBE buffer (89 mM Tris, 89 mM boric acid and 2.5 mM EDTA pH 8.0), containing 0.15 µg μl^{-1} of ethidium bromide.

Data Scoring and Analysis

The gels were visualized under ultraviolet (UV) light attached to a gel image analysis system which uses capillary gel electrophoresis to enable fast separation of nucleic acids based in size.

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This is to confirm the amplification and determine the background effect and primer dimers. The bands were scored for presence (1) or absence (0) of polymorphism for each marker on the basis of size in comparison with DNA ladder size maker of 25 - 500 bp thrice to minimize scoring errors. Alleles frequency base on Roger's genetic distance as reported in Sserumaga *et al*., 2014, was carried out. The generated data matrices were subjected to statistical analysis using R statistical software to determine the genetic diversity among the genotypes by cluster analysis. The polymorphism information content (PIC) value for each primer was obtained as the mean of the calculated PIC of all loci. The PIC for SSR was calculated as 1- Σpi^2 where pi is the frequency of i-th allele in a locus.

3. RESULTS AND DISCUSSION

Out of the 19 primers used in this study, only 10 primers showed polymorphism among the maize inbred lines (Table 3).

Table 3: Polymorphism of 19 primers among the 68 elite maize inbred lines from the breeding program of the Cereal Research Program of National Crop Resource Research Institute (NaCRRI)

S/N	Primers	Type of Bands
1	P-bnlg238	Polymorphic
2	Umc2038	Polymorphic
3	P-bnlg1014	Polymorphic
4	Umc2214	Polymorphic
5	P-umc2189	Polymorphic
6	Umc1447	Monomorphic
7	Phi037	Polymorphic
8	Phi034	No amplification
9	Umc1542	No amplification
10	Umc1862	Monomorphic
11	Phi115	Monomorphic
12	Bnlg434	Polymorphic
13	P-bnlg1179	Polymorphic
14	Bnlg1074	No amplification
15	Umc1545	Polymorphic
16	Umc1719	Monomorphic
17	Umc1432	Monomorphic
18	Umc2359	Monomorphic
19	Bnlg2190	Polymorphic

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Six of the primers showed monomorphic bands (Fig. 1)

A B

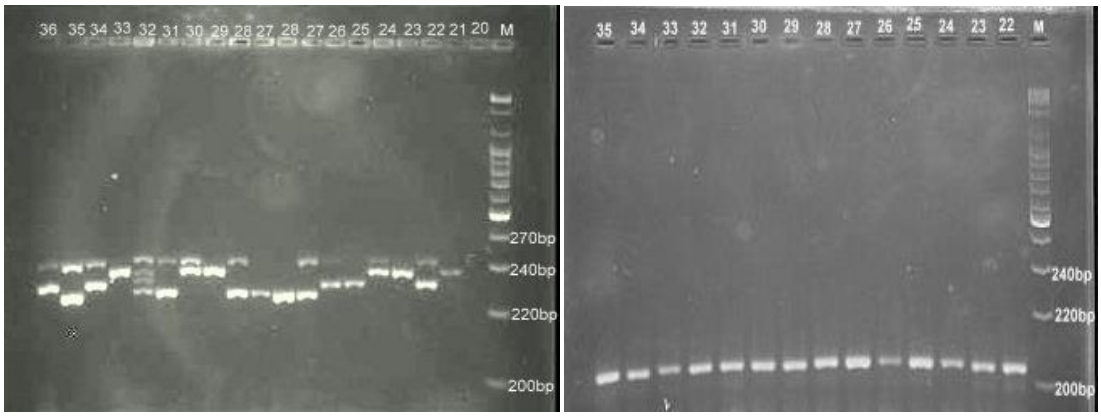

Fig. 1: PCR products of markers Umc2214 (A - polymorphic) and Bnlg434 (B - monomorphic) on maize inbred lines 20-36 on a 3% Metaphor agarose gel

while the remaining three primers did not amplify (Table 3). The presence of polymorphic bands obtained for the ten primers was in agreement with previous studies; P-bnlg1014, P-umc2189, P-bnlg1179 (Gemenet *et al*., 2010); Umc1545 (Mohammadreza, 2011); Bnlg434 (Nikolić *et al*., 2012); P-bnlg238, Umc2038, Umc2214, Phi037 and Bnlg2190 (Sserumaga *et al*., 2014).

The ten polymorphic primers among the 68 maize inbred lines revealed a total number of 33 alleles which ranged from 2 to 5 per locus and the highest number of alleles were found in Umc2214, P-bnlg238 and Umc2038 primers with 5, 4 and 4 alleles respectively (Table 4).

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Table 4: Bin location, allele numbers and polymorphic information content (PIC) of polymorphic primers among 68 elite maize inbred lines from the Maize Breeding Program of the Cereal Research Program of National Crop Resource Research Institute (NaCRRI), Namulonge, Uganda

Primer	Bin Location	Number of alleles	PIC
P-bnlg238	6.00	4	0.76
Umc2038	4.07	4	0.43
P-bnlg1014	1.01	3	0.49
Umc2214	2.10	5	0.60
P-Umc2189	1.00	3	0.19
Phi037	1.08	3	0.70
Bnlg434	7.01	3	0.13
P-bnlg1179	1.00	3	0.64
Umc1545	7.00	2	0.20
Bnlg2190	10.06	3	0.59
Mean		3.3	0.47

The number of alleles recorded in this study is different from what was obtained in previous studies of maize using SSR markers (Bantte and Prasanna, 2003; Reif *et al.*, 2003; Sserumaga *et al.*, 2014). This was most likely explained by the methodologies used in detecting the polymorphic markers which influenced allelic differences, uniformity of the inbred lines based on pedigrees, and the use of di- and tri- repeat types of SSR in this study. Dinucleotide SSR primers have been reported to yield a higher number of alleles per marker than SSRs with longer repeat motif (Choukan *et al.*, 2006; Adetimirin *et al.*, 2008).

The polymorphism information content (PIC) for the SSR loci obtained from this study ranges from 0.13 (Bnlg434) to 0.76 (P-bnlg238) with a mean of 0.47 (Table 4). Seven SSR loci (P-bnlg238, Phi037, P-bnlg1179, Umc2214, Bnlg2190, P-bnlg1014 and Umc2038) showed PIC values greater than 0.4 indicating their potential ability to detect differences among the inbred lines. This high PIC obtained for the seven markers is in agreement with the findings of Gemenet *et al*. (2010) and Sserumaga *et al*. (2014). PIC can be used to provide a measure of the genetic diversity and shows the discriminatory power of a marker by the number of alleles of the marker and the relative frequency of these alleles in the studied population (Senior *et al.*, 1998). This parameter measures the diversity of alleles in each gene locus. More than one band obtained on some of the inbred lines from the amplification process can be explained by the co-dominant nature of the SSR markers as reported in previous studies (Senior *et al.*, 1998; Matsuoka *et al.*,

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2002; Bantte and Prasanna 2003; Liu *et al*., 2003). The occurrence of double bands in maize has been attributed to differential drift of alleles at loci that were heterozygous in their derived line, amplification of similar sequences in different genomic regions due to duplication and the possible contamination of the line with pollen of another genotype.

The 68 maize inbred lines separated into four distinct phylogeny groups which is an indication of considerable genetic diversity in these inbred lines (Fig. 2).

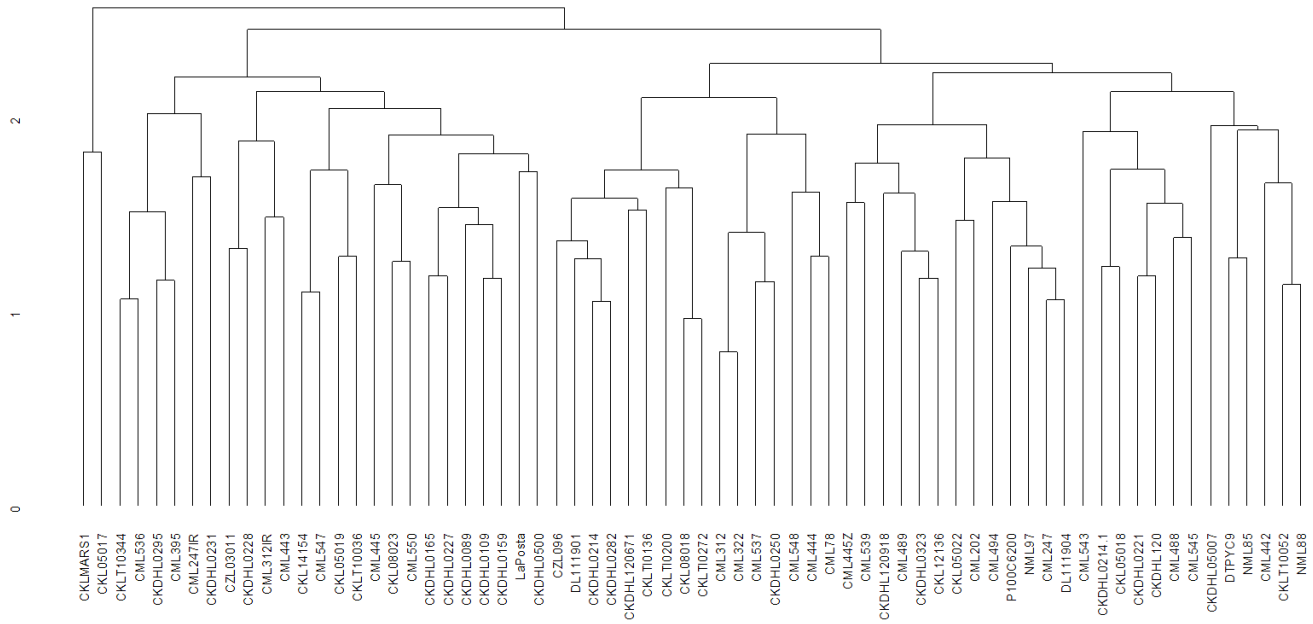

Fig. 2: Dendrogram showing diversity among the 68 elite maize inbred lines from the cereal research program of National Crop Resource Research Institute (NaCRRI)

The first cluster consisted of 26 closely related drought tolerant lines, the second cluster had 16 while the third and fourth clusters consisted of 13 lines. This generally agrees with maize accession origin records, for example, NML 85 and NML 88 which are of NaCRRI germplasm were grouped together in cluster 4 while the other NaCRRI maize germplasm; NML 97 was also grouped with the cluster 3 inbred lines. The highest genetic distance was found between inbred lines CKLMARS1F60677 and NML88 being the first and the last position on the dendrogram which originated from the CIMMYT and NaCRRI germplasm collections respectively. These groupings were also found to be consistent with their pedigree and origin since most of the CIMMYT and NaCRRI inbred lines were separately grouped (Table 1). Gerdes and Tracy (1994) showed that pedigree relationship can be used to test the effectiveness of markers in determining relationships among breeding lines. This observation demonstrates that SSRs are efficient

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markers to classify closely related lines and will reveal genetic associations that are reflective of the pedigree of the inbred lines (Smith *et al.*, 1997; Reif *et al.*, 2003; Legesse *et al.*, 2006). This information of genetic diversity within the drought tolerant maize inbred lines can be exploited for identification of possible inbred lines combinations and heterosis for the development of drought tolerant maize hybrids.

4. CONCLUSION

Markers P-bnlg 238, Phi037, P-bnlg1179 and UMC2214 were among the informative markers that significantly differentiated the maize inbred lines into groups. There was considerable genetic diversity among the sixty-eight elite maize inbred lines that can be exploited for the development of drought tolerant maize hybrids.

Abbreviation

SSRs, Simple Sequence Repeats**; QTL**, quantitative trait loci; **CTAB**, Cetyl trimethylammonium bromide; **PCR**, polymerase chain reaction; **UPGMA,** Unweighted Pair Group Method with Arithmetic Mean; **DNA,** deoxyribonucleic acid**; PIC,** Polymorphism information content; **MAS,** Marker-assisted selection; **MUARIK,** Makerere University of Agricultural Research Institute, Kabanyolo; **NaCRRI,** National Crop Resource Research Institute; **EtOH,** ethanol; **UV,** ultraviolet; **DfID,** Department for International Development; **CIRCLE**, Climate Impacts Research Capacity and Leadership Enhancement

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