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Molecular characterization of tropical maize inbred lines using microsatellite DNA markers

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Abstract

An insight on diversity and relationships among germplasm is important in any breeding program for crop improvement. The main objectives of our study were to: (i) determine the level of genetic diversity within mid altitude maize inbred lines resistant to weevils, aflatoxin accumulation and drought, (ii) to suggest potential heterotic groups using their genetic structures and distance based on cluster analysis with the aim to generate broad based source germplasm for mid altitude maize breeding program with combined traits of importance against Aflatoxin accumulation. In this study, 25 SSR markers were used to finger print forty two maize inbred lines to assess the genetic diversity, genetic relationships, and their population structure. A total of 184 alleles were identified at all the loci with an average of 7.36 and a range between two and 19 alleles per locus. The major allele frequency varied from 0.17 to 0.90 with an average of 0.49 while the minor allele frequency varied from 0.10 to 0.83 with an average of 0.51. The gene diversity values varied from 0.18 to 0.92 with an average of 0.65. Average heterozygosity percentage of the inbred lines was 4%, ranging from 0% to 2%, indicating the low level of heterozygosity within the inbred lines. The average polymorphism information content (PIC) was 0.61. A dendrogram constructed using unweighted Neighbour Joining algorithm suggested three heterotic groups among the inbred lines. The three heterotic patterns based on the SSR markers need to be verified by field testing to confirm what appears to be promising alternative heterotic patterns. The fixed pattern detected using SSR markers could potentially contribute towards effective utilization of the inbred lines for the exploitation of heterosis and formation of genetically diverse sources population.

Keywords: maize, microsatellites, genetic diversity, population structure, inbred line, heterotic group, molecular markers

Introduction

Maize (*Zea mays* L) currently covers approximately 30 million ha of arable land in Sub-Saharan Africa (SSA). Current maize yields are very low (about 1.8 t ha^{-1}) relative to the existing potential. Maize serves both as a staple food crop and non-traditional cash crop, with maize exports earning over 90 million US dollars in 5 years up to 2007 in Uganda (UBOS, 2007). Per capita consumption ranges from 25-85kg depending on the region. Additionally, about 77% of maize grain is used as food in SSA in countries outside South Africa (DTMA, 2012). Uganda's potential maize export capacity in the region is estimated at between 100,000-150,000 MT per annum. Maize serves as one of the most important staple cereal food crop in Uganda, as it ranks first both in production and consumption (UBOS, 2009). The land area under its production increased from 750,000 ha to 862,000 ha from 2004 to 2008. During the same period, production increased from 10,080, 000 tonnes to 1,266,000 tons (UBOS, 2009). Of the estimated national annual output, post-harvest losses claim 10%, 25% is retained for on-farm consumption, 50% is consumed by non-producers of maize within the country, and the remaining 15% is available for export (PSF, 2003). Uganda's potential maize export capacity in the region is estimated at between 100,000-150,000 MT per annum. The area planted to maize is anticipated to further increase with the increase in the population. An additional corresponding increase in yield in the future will require an efficient breeding program with well-established germplasm in order to exploit potential heterosis in germplasm development. Despite this, most breeding program in the past mainly targeted on production of high yielding varieties, with resistance to gray leaf spot (GLS; *Cercospora zeae-*

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maydis Tehon), maize streak virus (MSV), turcicum leaf bright (TLB; *Exserohilum turcicum*) and Insects (Weevil and stem borers), then drought tolerance and low nitrogen use efficiency. The maize breeding program in Uganda has limited genetically diverse maize germplasm and has not developed high yielding Aspergillus ear rot resistant hybrid and Open-pollinated varieties for the most of the households who are exposed to consumption of high levels aflatoxins in maize.

In any breeding program, information and utilization of knowledge on the genetic diversity and of relationship among the breeding material has significant impact on the identification of promising hybrid combinations to exploit heterosis and inbred line development hence establishment of heterotic groups for use as source materials in breeding program. The process of identifying lines to produce outstanding single crosses as quickly as possible is dependent upon procedures such as field evaluation of diallel and topcrosses and the use of pedigree information. and dependence on the morphological traits. The methods utilize morphological traits and are slow, laborious, greatly influenced by environment and sometimes the information about the pedigree of the lines is not available. The advancement in use of molecular markers has proven valuable for genetic diversity analysis at the DNA level in plant species (Melchinger and Gumber, 1998). Unlike the morphological markers, molecular markers are not influenced by environmental factors; thus they reflect the actual level of genetic difference existing among the genotypes (Westman and Kresovich, 1997; Legesse et al, 2006). Assignment of inbred lines to heterotic groups using molecular markers allows the characterization of a greater number of lines, hence potentially increasing the efficiency of maize breeding programs (Choukan et al, 2006; Reif et al, 2003). Grouping lines to different heterotic groups avoids the development and evaluation of the crosses that would be eventually be discarded (Choukan et al, 2006; Terron et al, 1997). Microsatellites (Litt and Luty, 1989), also known as simple sequence repeats (SSRs) (Tautz et al, 1986), short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs) (McDonald and Potts, 1997), are the smallest class of simple repetitive DNA sequences, or are DNA markers with short stretches of tandemly repeated di-, tri-, or tetra-nucleotide motifs (Weber, 1990; Legesse et al, 2006). Some authors (Armour et al, 1999) define microsatellites as 2–8 bp repeats, others (Goldstein and Pollock, 1997) as 1–6 or even 1–5 bp repeats (Schlotterer, 1998). In this study we used SSR DNA markers mainly because they are characterized by a great abundance (Matsuoka et al, 2002), High variability and even distributed throughout a wide range of genomic regions (Legesse et al, 2006). They are co-dominant, highly polymorphic, multi-allelic and became the marker of choice for genetic analysis in crops (Gupta and Varshney, 2000) although single nucleotide polymorphism (SNP) markers are now widely used. The use of microsatellites have proved to be a valuable tool not only for genome mapping (Taramino and Tingey, 1996) but also population and conservation genetic studies (Powell et al, 1996), property right protection (Kubik et al, 2001), marker-assisted selection (Weising et al, 1998) and diversity measurements (Pinto et al, 2003). They have also substantiated their use in assigning lines into heterotic groups (Enoki et al, 2002). In terms of power to discriminate, SSRs have proved better than restriction fragment length polymorphism (RFLP) markers and can reveal genetic associations that are reflective of the pedigree of the inbred lines (Legesse et al, 2006). Comparative studies the effectiveness of different maker systems for estimating genetic similarity among maize inbreds showed that SSR makers revealed the highest level of polymorphism per single locus, due to their codominant natures and high number of alleles per locus (Pejic et al, 1998). SSR technology is dependent on polymerase chain reaction (PCR); therefore, polymorphism can be detected by using the less costly and more widely available agarose system (Bantte and Prasanna, 2003).

In this study, maize inbred lines obtained from the International Maize and Wheat Improvement Center CIMMYT in Kenya and Mexico, the International Institute of Tropical Agriculture (IITA) in Nigeria and the Cereals Program of the National Agriculture Research Organization (NARO) of Uganda were genotyped using SSR markers. DNA polymorphisms were detected by separation using the QIAxcel system which uses capillary gel electrophoresis to enable fast separation of nucleic acids based in size. The specific objectives of the study were to i) determine the level of genetic diversity within mid altitude and subtropical maize inbred lines resistant to weevils, aflatoxin accumulation, and tolerance to drought; ii) to suggest potential heterotic groups based on their genetic structures.

Materials and Methods

Plant materials

A total of 41 inbred lines including 24 weevil resistant lines from the NARO maize breeding program in Uganda, 13 Aspergillus ear rot resistant and three drought tolerant inbred lines from CIMMYT and one Aspergillus ear rot resistant from IITA, developed in collaboration with USDA-SR ARS were used in thisstudy. The 24 weevel resistant inbred lines were developed from crosses between the best weevil resistant NARO lines and the best drought tolerant mid-altitude CIMMYT lines. They were selected for disease resistance mainly MSV, GLS, and TLB and also for storage weevil resistance. The inbred lines form CIMMYT Kenya and IITA are resistant or tolerant to *Aspergillus flavus* and *parasiticus*. The pedigree of these lines is given in Table 1.

Table 1 - List of inbred lines used in the study.

No	Identification	Original Pedigree	Origin
1	WL 118-1-1	[WEEVIL/CML197]-B-13-B-B-B-B	NARO
2	WL 118-3	[WEEVIL/387]-B-19-B-B-B-B-B-B	NARO
3	WL 118-6	[WEEVIL/CML390]-B-19-B-B-B-B	NARO
4	WL 118-9	[WEEVIL/COMPE20]-B-26-B-B-B	NARO
5	WL 118-10	[WEEVIL/CML202]-B-7-B-B-B-B	NARO
6	WL 118-11	[WEEVIL/CML205]-B-24-B-B-B-B	NARO
7	WL 118-16	SZSYNA99-F2-79-2-3-B-B-B	NARO
8	WL 118-17	SZSYNA99-F2-81-4-2-B-B-B-B	NARO
9	WL 429-8	[CML312/MAS[MSR/312]-117-2]-B-50-B-1-B-B	NARO
10	WL 429-12	[CML312/MAS[MSR/312]-117-2]-B-91-B-B-B-B	NARO
11	WL 429-16	[WEEVIL/CML197]-B-9-B-B-B-B	NARO
12	WL 429-18	[WEEVIL/CML197]-B-12-B-B-B-B	NARO
13	WL 429-19	[WEEVIL/CML197]-B-18-B-B-B-B	NARO
14	WL 429-14	[WEEVIL/CML444]-B-22-B-B-B-B	NARO
15	WL 429-24	[WEEVIL/CML312]-B-1-B-B-B-B	NARO
16	WL 429-26	[WEEVIL/CML312]-B-23-B-B-B-B	NARO
17	WL 429-27	[WEEVIL/CML312]-B-32-B-B-B-B	NARO
18	WL 429-30	[WEEVIL/CML387]-B-8-B-B-B-B	NARO
19	TZAR504	(GT-MAS:gk/*2/ KU1414SR)-8-1-2-3-B*7	IITA
20	WL 429-33	[WEEVIL/CML389]-B-5-B-B-B-B	NARO
21	WL 429-35	[WEEVIL/CML389]-B-11-B-B-B-B	NARO
22	WL 429-36	[WEEVIL/CML389]-B-15-B-B-B-B	NARO
23	WL 429-38	[WEEVIL/CML389]-B-17-B-B-B-B	NARO
24	WL 429-39	[WEEVIL/CML389]-B-18-B-B-B-B	NARO
25	WL 429-43	[WEEVIL/CML389]-B-24-B-B-B-B	NARO
26	CML247	(G24F119*G24F54)-6-4-1-1-BB-f	CIMMYT
27	CML495	[P NVA. BCO.(S/D)xNPH-28]F32-B-1-B-1-2-BBBBBB	CIMMYT
28	CML264	Pob21C5F219-3-1-B-##-8-1-3-BBB-f	CIMMYT
29	POB.501	POB.501c3 F2 20-3-1-2-B-B-B-B	CIMMYT
30	MIRTC5	MIRTC5 Bco F78-2-2-1-1-1xDERRc2 15-3-7-1-1-B-B-B	CIMMYT
31	P502	P502c2-185-3-4-2-3-B-2-B-B-B-B-B	CIMMYT
32	CML348	G26SEQC3-H83-1-1-2-1-B	CIMMYT
33	CL-RCW31	CL-RCW31 (CML-247*CL-G2415)-B-1-B-2-1-1-BB-B-B	CIMMYT
34	CL-RCW37	CL-RCW37	CIMMYT
35	La Posta Seq C7	La Posta Seq C7-F103-2-1-1-1xMIRTC5 Bco F80-4-2-1-1-1-3-1-B-B	CIMMYT
36	CL-RCW35	[CL-04317*v]-1-B-1-1-2-BBBB	CIMMYT
37	CL-02510	P25C5HC246-3-1-BB-2-#-BBBBBBB	CIMMYT
38	CML451	[NPH28-1*G25)*NPH28]-1-2-1-1-3-1-B*6	CIMMYT
39	CML202	ZSR923S4BULK-5-1-b-b	CIMMYT
40	CML444	P43C9-1-1-1-1-1-BBBB	CIMMYT
41	CML322	89[L/LMBR]17-B-5-3-1-4-B*4	CIMMYT

DNA Isolation

Kernels for each inbred lines were ground and with mortar and Geno/Grinder (Spex sample Prep). Genomic DNA was isolated using IncloneTM Genomic DNA prep kit as described in the manual. In brief, 100 mg of kernel powder for each inbred lines was added in 1.5 ml microcentrifuge tube containing 600 µl of lysis buffer (ICL) and vortexed for 2 minutes then incubated for 65°C for 30 min while inverting the sample at an interval of every 10 minutes. Added 1.5 µl of RNase A (4 mg ml^{-1}) and mixed the solution well by vortexing. Extracted DNA was electrophoresed on 1.1% (w/v) superfine agarose gels (Amresco), stained with LoadigSTARTM solution (DYNEBIO), and photographed under UV light attached to gel image analysis system (Core Bio, ImaxTM)

PCR conditions and electrophoresis for SSR analysis

A total of 25 SSR maize primers were chosen from the MaizeGDB database (http://nucleus.agron.missouri.edu/cgi-bin/ssr_bin.pl) on the basis of bin location (to maximize genomic coverage) and avoidance of di-nucleotide repeats because of the difficulty in accurately size alleles plus also with references from other publications (Legesse et al, 2006; Choukan et al, 2006) and used for PCR amplification of the 41 inbred lines. They were all selected based on the bin locations, which provides a uniform coverage of all the ten chromosomes in the maize genome. Primer names and chromosome loci (Bin number) of the SSR loci evaluated are presented in Table 2. Oligonucleotide primers were synthesized at nano-mole concentration by Integrated DNA Technologies Leuven, Belgium (IDT) Primer Company.

All oligonucleotide primers were diluted to a working concentration of 10 µM with sterile water and

Table 2 - Information on the 25 SSR loci used in this study.

No	Marker Type	Repat	Bin No	Ann Temp	Major Allele Frequency	Minor Allele Frequency	Genotype No	Number of Alleles	Gene Diversity	Observed Heterozygosity	PIC
1	Bnlg1082	AG(11)	9.02	60	0.37	0.63	7	6	0.74	0.02	0.70
2	Bnlg1762	AG(15)	10.03	60	0.56	0.44	8	8	0.63	0.12	0.59
3	Bnlg2190	AG(31)	10.06	65	0.17	0.83	20	19	0.92	0.02	0.91
4	Bnlg238		6	65	0.29	0.71	14	13	0.85	0.05	0.84
5	Bnlg602		3.04	65	0.39	0.61	10	10	0.79	0.00	0.78
6	Nc003	AG	2.06	55	0.37	0.63	9	9	0.77	0.00	0.74
7	phi015	AAAC	8.08	62	0.75	0.25	4	4	0.41	0.00	0.38
8	phi032	AAAG	9.04	65	0.71	0.29	3	3	0.45	0.00	0.40
9	phi034	CCT	7.02	60	0.46	0.54	10	9	0.72	0.02	0.69
10	phi037	AG	1.08	60	0.22	0.78	14	13	0.86	0.02	0.85
11	phi079	AGATG	4.05	65	0.51	0.49	9	9	0.70	0.00	0.68
12	phi085	AACGC	5.06	65	0.61	0.39	4	4	0.57	0.00	0.52
13	phi109275	AGCT	1.03	55	0.39	0.61	6	6	0.77	0.00	0.74
14	phi115	AT/ATAC	8.03	65	0.32	0.68	10	9	0.79	0.02	0.77
15	phi427434	ACC	2.08	55	0.27	0.73	5	5	0.79	0.00	0.75
16	Umc1153	(TCA)4	5.09	65	0.29	0.71	7	7	0.79	0.00	0.76
17	Umc1296	(GGT)7	6.06	65	0.49	0.51	6	6	0.65	0.00	0.60
18	Umc1367	(CGA)6	10.03	65	0.90	0.10	2	2	0.18	0.00	0.17
19	Umc1568	(TAG)4	1.02	55	0.51	0.49	5	5	0.62	0.00	0.56
20	Umc1669	(AGA)4	4.01	65	0.90	0.10	2	2	0.18	0.00	0.16
21	Umc1677	(GGC)4	10.05	60	0.66	0.34	5	4	0.51	0.03	0.46
22	Umc2036	(GTC)4	5.01	65	0.77	0.23	5	4	0.38	0.20	0.35
23	Umc2038	(GAC)4	4.07	65	0.48	0.52	10	7	0.66	0.17	0.61
24	Umc2050	(CGC)4	3.07	65	0.65	0.35	8	5	0.54	0.15	0.50
25	Umc2214	(CTT)4	2.1	65	0.22	0.78	17	15	0.87	0.10	0.86
	Mean				0.49	0.51	8.00	7.36	0.65	0.04	0.61

stored at -20°C. PCR reactions were performed in 30 µl volumes containing 2 µl of 10ng μl^{-1} template DNA, 1.2 pmols each of primers, 3 µl of 10x PCR buffer, 0.6 µl of 10 mM of dNTP, and 0.3 units of Taq polymerase (IncloneTM). The reactions were performed with a Peltier Thermal Cycler (MJ Research Inc, Watertown, MA) using the amplification conditions of 94°C for 3 min, followed by 34 cycles of 98°C for 10 sec, A°C for 30 sec and 72°C for 1 min and 40 sec, followed by extension of 72°C for 7 min. A°C refers to the appropriate annealing temperature of the primers as in Table 2. PCR profiles with annealing temperatures ranging from 55 to 65°C were used for different SSR primers (Table 2). The PCR products were electrophoresed on 3% (w/v) superfine agarose gels (Amresco). The PCR products were stained with loadingSTARTM dye and photographed under UV light attached to a gel image analysis system (Core Bio, ImaxTM) to confirm the amplification and also determine the back ground effect plus primer dimers. Later, the QIAxcel system which uses capillary gel electrophoresis to enable fast separation of nucleic acids based in size was used to finely separate the fragments. Allele sizes of amplified fragments were scored on the basis of size in comparison with DNA size maker of 25 - 500 bp.

SSRs data scoring and statistical analysis

The SSR bands were scored using QIAexcel biocalculator software which determines the exact band size of each peak (capillary electrophoresis) producing allelic data. The allelic pattern of each inbred line was also cross-checked also with agarose gel electrophoresis. Allele calls were automatically made when a peak from a data samples matched the location of a bin. Completed results were run in Allelobin software (Prasanth et al, 1997) to correct any errors in the scored alleles resulting from slippage of DNA polymerase during PCR resulting into stutter peaks (Schlotterer and Tautz, 1992). Computation of allele frequency-based Roger's genetic distance (Rogers, 1972) was carried out using PowerMarker version 3.25 (Liu and Muse, 2005) and used for cluster analysis. A dendrogram was constructed using the neighbor-Joining algorithm implemented in PowerMarker and the resulting trees were visualized with MEGA version 5 software (Tamura et al, 2007).

Results and Discussion

SSR polymorphism

Analysis of all the 25 marker loci revealed polymorphism across the 41 inbred lines. A total number of 184 alleles were detected with a range from 2 (umc1367 and umc1669) to 19 (Bnlg2190) alleles per locus and an average of 7.36 alleles (Table 2), values different to previous studies using SSRs on maize inbred lines (Warburton et al, 2002; Bantte and Prasanna, 2003; Reif et al, 2003; Armour et al, 1999). Differences in the numbers of alleles between studies could be explained mainly due to the size of the samples under study, the methodologies employed for detection of polymorphic markers which influence allelic differences, expected diversity or uniformity based on pedigrees, and most importantly, use of di- tri- and tetra-repeat types of SSR in the present study. Dinucleotide SSR primers are known to yield a significantly higher number of alleles per marker than SSRs with longer repeat motif and also they are often not used in general because of the difficulty in accurately sizing alleles (Heckenberger et al, 2002; Choukan et al, 2006; Adetimirin et al, 2008).

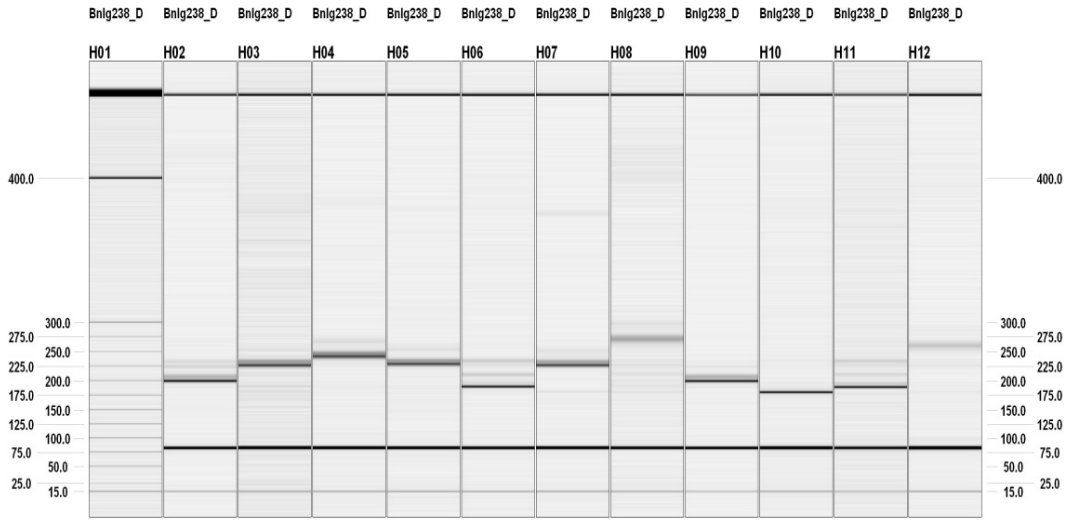

Figure 1 - Capillary electrophoresis polymorphic data for genetic diversity with inbred lines produced using QIAxcel system.

Polymorphism information content (PIC) value

The PIC value of the SSR loci ranged from 0.16 (umc1669) to 0.91 (bnlg2190) with the average of 0.61 (Table 2). Fourteen SSR loci (bnlg1082, bnlg2190, bnlg238, bnlg602, nc003, phi034, phi079, phi109275, phi115, phi427434, umc1153, umc1296, umc2038 and umc2214) manifested PIC values over 0.6 indicating their potential informativeness to detect differences among the inbred lines.

Earlier reports indicated that for 70 SSR markers in 94 inbred lines representative of the genetic diversity among lines derived from the Corn Belt Dent and Southern Dent Maize races (Senior et al, 1998). These results, together with the high mean genetic distance among the genotypes, indicate considerable diversity among inbred lines tested in this study. The PIC value demonstrates the informativeness of the SSR loci and their potential to detect differences among the inbred lines based on their genetic relationships. In overall, dinucleotide SSR loci identified the largest mean number of alleles (19) and mean PIC (0.91) as compared to tri, and tetra nucleotide repeats in this study, which is also in close agreement with previous observations for maize (Senior et al, 1998; Enoki et al, 2002; Adetimirin et al, 2008).

In this study, capillary gel electrophoresis was used for to score microsatellites as compared to polyacrylamide gel and agarose gel analysis; this is not the most routinely used technology for analysis in previous studies as it is more costly than polyacrylamide and agarose gel system. However, this automated detection system is able to resolve allelic variation better than agarose gel electrophoresis analysis, and consequently, the number of alleles obtained are higher than that reported in other studies. This may be particularly important for SSR loci containing dinucleotide repeats whose amplification products are in the 130 to 200 bp range, because PCR products differing by two base pairs cannot be resolved well with agarose gel (Senior et al, 1998; Sibov et al, 2003).

As reported in a study by Bantte and Prasanna (2003), several inbred lines revealed more than one band during amplifications, which may have resulted from the co-dominant nature of the SSR markers. In the present study, this was particularly evident in the bnlg228 loci, which revealed high frequencies of double bands (Figure 1). Similar results have been previously reported in maize inbred lines (Senior et al, 1998; Matsuoka et al, 2002; Liu et al, 2003) These authors speculated on a number of probable causes for the occurrence of double bands in maize, including differential drift or fixation of alleles at loci that were heterozygous in the plants from which the line was derived (residual heterozygosity), contamination of the line with pollen or seed of another genotypes, mutation at specific SSR loci, or amplification of similar sequences in different genomic regions due to duplication. The most plausible explanation for obtaining such results in our case could be residual heterozygosity, as a result of small number of generations of inbreeding.

The ability to provide distance measures between the inbred lines that reflect pedigree relatedness ensures a more stringent evaluation of the adequacy of a marker profile data. The fact that minimum genetic distance was revealed between WL 118-16, WL 11817, WL 429-18, and WL 429-19 (Figure 2) is a good indication confirming the power of SSR markers to distinguish between closely related inbred lines (Smith et al, 1997).

Heterozygosity

The average heterozygosity was 4% among the markers used in the study with a range from 0% to 20% (Table 2). In this study SSR marker umc2036 showed the highest heterozygosity (20%) in the study. This reflected that there was greater fixation of the inbred lines regardless of their diversity. Gene diversity ranged from 0.92 (bnlg2190) to 0.18 (umc1669) with

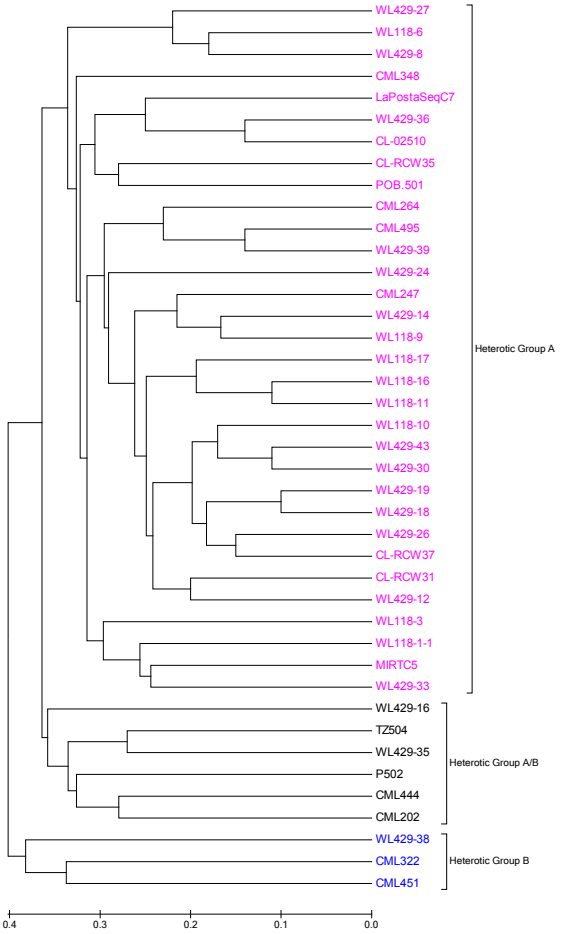

Figure 2 - Neighbor-Joining tree for 41 inbred lines based on Roger's genetic distance.

an average of 0.65 (Table 2). Gene diversity is equivalent to the expected heterozygosity for diploid data and it is defined as the probability that two randomly chosen haplotypes (alleles) are different in the sample. The average genetic diversity detected among all the inbred lines indicated that high levels of polymorphisms in the inbreds. These results are in close agreement with the findings reported in studies with maize inbred lines using a SSR marker system (Smith et al, 1997; Senior et al, 1998; Vaz Patto et al, 2004).

Genetic distance and cluster analysis

Genetic distance ranged from 0.20 to 0.989. A total of 10 pairs of lines (out of 41) had genetic distance greater than 0.9, indicating that they exhibited differences at 25 SSR loci studied. The Neighbor-Joining tree based on Roger's genetic distance computed from 25 SSR loci suggested three groups among the inbred lines (Figure 2) meaning within each group, distance was > 4% genetic distance belonged to the same group.

Overall, this study revealed that SSR markers largely separated the inbred lines into different cluster, which generally agrees with the pedigree records and adaptation regimes. For example CML202 and CML444 belong to the same transitional heterotic A/B group but skewed toward heterotic group B. Also CML322 and 451 all in the same B group according to CIMMYT grouping. High overall genetic diversity (0.65) among the inbred line combinations indicates an opportunity to exploit the inbred lines for the development of varieties and start point of pedigree breeding population used to produce promising inbred lines.

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