Microsatellite Marker Application in the Assessement of Sorgum Genetic Diversity

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Abstract**—** Molecular marker information supported by quality phenotypying facilitates the choice of suitable parents for applied breeding. We used twenty microsatellite markers that are reasonably distributed across the sorghum genome to screen selected sorghum genotypes for bioenergy characteristics. The differences among the genotypes were significant and the mean polymorphic information content was 0.379. Construction of a phylogenetic tree with the molecular data using Darwin revealed clusters of genotypes with similar phenotypic characteristics. Most TS1 and Wray genotypes, which constitute the sweetest genotypes in this study are centrally located on the tree while the genotypes with less stalk-sugar are found towards the edge`. On average, the performance of the twenty microsatellite markers was good because it yielded useful molecular information on the sorghum genotypes. Polymorphic information content and heterozygosity values of the markers provide insight into the usefulness of individual markers that can be included in future molecular studies involving sorghum breeding.

Keywords**—**Bioenergy traits, Biofuel feedstock, Sorghum

I. Introduction

OVER the years, a variant of sorghum (*Sorghum bicolor* (L.) Moench) commonly known as sweet sorghum is one crop that has gained importance in several sub-Saharan countries. Many reasons account for the increased interest in the crop but the most important is its possible use as a candidate biofuel feedstock. The global search for alternative fuel sources that are sustainable and friendlier to the environment has led to the consideration of a number sugar producing crops. Brazil's success with sugar cane as an alternative biofuel feed stock only heightened the interest in sweet sorghum research owing to the crop's advantageous attributes over sugar cane [1]. Sweet sorghum or 'sorgos' belong to the same domesticated species as grain sorghum and therefore share in some of the favorable characteristics of grain sorghum such as wide adaptability, saline-alkaline tolerance, rapid growth and high biomass [2]. In addition, sweet sorghum has been shown to have the potential to accumulate an amount of sugar in their stalks approximating that of sugar cane (*Saccharum spp*) [3]. However, the growing interest and preference for sweet sorghum as a more suited biofuel feedstock for most sub- Saharan countries compared to sugar cane lies in its multi-purpose nature. In a number of third-world countries where sorghum is grown but is not the staple crop, such as in the Southern parts of Africa, the development of the crop as an alternative fuel source is even more attractive. In these countries, the growing of the crop is practiced by mainly resource-poor farmers in the marginal regions where the staple crop cannot survive [4]. Therefore, food versus fuel concerns that are associated with sorghum's proposed use as a fuel feedstock are considerably lessened.

Sweet- stalked variants of sorghum originated from China as land races and have been subjected to trait improvement mainly through conventional breeding for a long time [3]. Typical of conventional breeding, sorghum breeding programmes have been long, labour intensive and marked by slow progress. By the year 2010, the crop had received comparatively little application of advanced breeding technology [5]. Today, with the heightened interest in the potential of sweet sorghum as a multi-purpose crop for food, feed and fuel, application of newer technology in breeding is essential to facilitate progress.

In this study, microsatellite markers are applied to investigate the genetic relatedness of twenty sorghum genotypes and evaluate the ease of use of microsatellite marker technology.

II. Procedure

A. Composition of the Study

The study comprised of two main experiments namely, field study, and the green house and laboratory experiments. Phenotypic data collected from the field experiment and from previous years' studies on the genotypes was used to back-up molecular data from the laboratory

B. Field Experiment

This was carried out in 2011-2012 at the University of Zambia field station (15˚23"S,28˚20"E, 1261m above sea level and deep brown to yellowish red sandy loam to clay loam soil type). Plant materials made up of parents, crosses, and backcrosses were planted and data on six parameters brix (B), plant (PH) height, plant girth (PG), midrib score (MRSC), panicle weight (PW), juice volume (JV) was collected. The plant materials used are shown in Table I. The seventh and

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eighth parameters namely, stem biomass per hectare (STMBHA) in kg/ha and total sugar per hectare (TOTSUG) also in kg/ha were derived from the measured parameters using the formulas;

STMBHA = (1ha x weight of five stems)/Area of stems
TOTSUG= (1ha x mass of juice for five stems)/Area of stems

TABLE I
DESCRIPTION OF THE SWEET SORGHUM MATERIALS USED IN THIS STUDY

Genotype	Description	Pedigree
Wray 2.5	short plant, sweet stem	Parent
Sima	moderately sweet	Parent
Cowley	moderately sweet	Parent
TS1 PRT	early aerial tillers	Parent
Lusitu	tall, local variety	Parent
Praj crosses	stay green	F3
W2.2.3	prone to stem borers	F3
Wray mwp	tall plant, stay green	F3
WxL crosses	tall plants, wide girth	F1
TS1 1.4.4	healthy stems, wide girth	F3
TS1 1.3.8	healthy stems	F3
TS1 1.3.6	short internodes, prop roots	F3
TS1 1.4.5	many prop roots	F3
WBC28	prone to stem borers	Backcross 1
WBC1	prone to stem borers	Backcross 1
TSBC1	short plant, long internodes	Backcross 1

C. Green House and Laboratory Experiment

A duplicate of the genotypes in the field experiment was grown in pots in the green house. At three weeks, young leaves from the green house plants were taken for total genomic DNA isolation. Graham's [6] CTAB method with very minor modifications, mainly pertaining to incubation temperatures and periods was used. The DNA extracted was quantified using a Thermal Scientific Nanodrop 2000 spectrophotometer before commencing DNA dilution. The GP1 primer [7] with the nucleotide sequence shown in Table II was used in the initial optimization process. The reaction mixture consisted of 8µl of DNA solution (40ng/µl), 2µl 10x buffer,1µl GP1 primer mix (10µM), 0.5µl deoxyribonucleotide triphosphates (5nM each), 0.25µl of 5U/µl Taq polymerase enzyme and 11.75µl double distilled water. The total volume of the reaction mixture was 20µl. Temperatures in the range 55-60˚C were tested along with several different PCR conditions in the initial optimization process of the GP1 primer. Successful amplification was achieved at 60˚C annealing temperature and 25 cycles of the following PCR program.

Initial denaturation at 95˚C for 10:00 minutes
Denaturation at 95˚C for 00: 15minutes
Annealing at 60˚C for 01:00 minutes
Elongation at 72˚C for 20:00 minutes
A final holding temperature of 4˚C

Upon achieving successful amplification using the GP1 primer, twenty microsatellite markers covering the entire sorghum genome (at least one marker for each of the ten sorghum chromosomes) were used in the genetic diversity assessment of the sorghum genotypes in the study. The master mix contained 1x PCR buffer, 0.4µl of 2mM magnesium chloride, 0.8µl of 0.16mM deoxyribonucleotide triphosphates, 0.2µl of 0.04pmol forward primer, 1µl of 0.2pmol reverse primer, 0.04µl of 0.2U Taq polymerase, 2.76µl of sterile water and 0.8µl of 0.16pmol fluorescent label. A 10.0µl final volume, made up of 3.0µl of DNA and 7µl of master mix was used with 40 cycles of the following PCR program.

Initial denaturation at 94˚C for 05:00 minutes
Denaturation at 94˚C for 00:30 minutes
Annealing at 50˚C for 01:00 minutes
Elongation at 72˚C for 20:00 minutes
A final holding temperature of 4˚C

TABLE II
PRIMER SEQUENCES USED TO AMPLIFY THE SUCROSE SYNTHASE GENE FRAGMENT.

Forward:	5'GCGTCGACCCAAGAGCTTGGTTTGGAGAAGG 3'
Reverse:	5'GCTCTAGACTGTGAACTGGATGAGAAGTGG 3'

D.Analysis

Field experiment data was analyzed using the 14th edition of GENSTAT while molecular data from the green house and laboratory experiment was analyzed using Gene Mapper version 4, Power Marker version 3.25 and Darwin version 5.

III. RESULTS

A. Phenotypic Data

The field experiment revealed significant phenotypic differences among the sorghum genotypes in the study in terms of all the measured parameters as well as the derived parameters (Table III). TS1 1.4.5, a tall and sweet genotype had the highest brix percent. Praj.1.9.1-24 was the genotype yielding the lowest brix percent.

B. Initial Optimization with GP1 Primer

The PCR at the reported literature value of 56˚C annealing temperature yielded no visible band, but when the temperature was raised to 60 ˚C, an approximately 1kbp band was obtained for all genotypes in the study.

A. Genetic Diversity with Microsatelite Markers

Screening of the genotypes with the twenty microsatellite markers produced several differently sized fragments for most of the markers in the study as shown in Fig. 1.

In Table IV, marker xtxp145 had the highest polymorphic information content (PIC) followed by xtxp141. Four markers out of the twenty had a PIC of 0.00.

Eight of the markers had a PIC above the mean PIC while the rest of the markers had their PICs below the mean. The mean heterozygosity and gene diversity across the genotypes was 0.0838 and 0.3731 respectively. The marker xtxp145 had the highest heterozygosity and gene diversity values. Marker SbAG02 had the highest major allele frequency among all the twenty markers used in the study.

TABLE III
MEAN PERFORMANCE OF THE GENOTYPES FOR BRIX AND OTHER BIOENERGY TRAITS

Genotype	B (%)	PH m	PG cm	MRSC	GW kg	PW kg	JV ml
Cowley	10.64	1.960	6.300	1.700	0.270	0.070	13.4
Wray mwp	13.28	3.050	7.800	3.900	1.200	0.240	144.0
P1.9.1-24	6.64	2.700	7.120	2.600	0.780	0.190	31.0
Sima	11.00	2.280	7.020	2.500	0.582	0.066	57.0
P2.1.2	6.56	2.020	7.820	3.200	0.480	0.136	18.4
TS1 1.3.6	12.24	1.720	6.980	2.600	1.720	0.124	53.0
TS1PRT	12.26	2.620	6.960	3.000	0.800	0.200	56.0
TS1 1.4.4	12.04	2.210	7.640	1.600	0.870	0.110	88.2
TS1 1.4.5	17.14	2.400	7.100	2.500	1.090	0.192	54.8
TSBC1	11.38	1.460	7.080	4.200	0.610	0.052	57.6
TS1 1.3.8	13.14	1.720	6.260	3.500	0.800	0.120	61.0
Lusitu	12.56	1.440	6.500	4.300	0.750	0.160	22.4
W2.2.3	10.28	1.618	5.620	3.600	0.370	0.094	28.8
Wray2.5	6.44	1.240	6.760	4.200	0.440	0.098	73.6
WBC28	13.00	1.880	6.380	4.400	0.318	0.044	36.4
WBC1	10.76	1.900	6.020	2.500	0.386	0.064	21.6
WxL5	14.72	2.680	7.720	3.000	1.070	0.138	48.4
WxL8	12.54	2.226	6.320	3.800	0.420	0.180	31.0
CV (%)	24.3	17.5	11.3	20.6	69.9	63.9	53.5
L.s.d	3.4900	0.2410	0.5160	0.4337	0.3348	0.05376	17.72

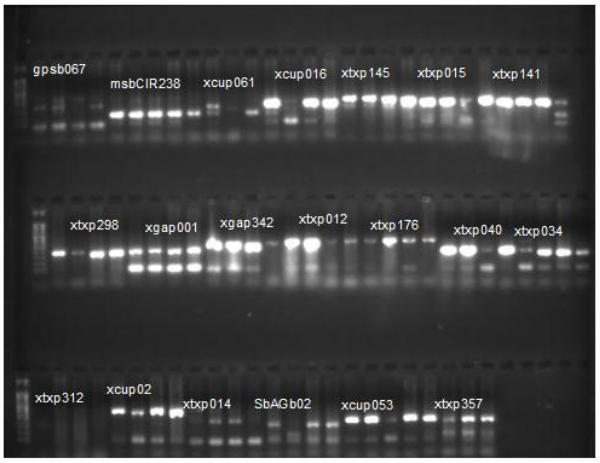

Fig.1 PCR products for sorghum genotypes using 20 different SSR markers.

There were quite a number of markers that had several alleles. These along with xtxp034, a marker with a high PIC and four different alleles across all the genotypes are responsible for the observed sorting and clustering of the genotypes in the study.

The most common allele across all the genotypes considering all the twenty markers belongs to the marker SbAG02. The 113 base-pair allele for this marker was closely followed by the 216 base-pair allele of the marker xcup02. Other markers with alleles of high frequencies above 0.7 include xcup061, xcup053, and xtxp040.

B. Factorial Analysis and Phylogenetic Tree Construction

In Fig. 2, factorial analysis using Darwin placed the Praj1 and Cowley genotypes in the same quadrant of the two axes. The rest of the genotypes were scattered in the remaining three quadrants except for TS1 1.4.4 which is on the border line between two quadrants.

A phylogenetic tree (Fig. 3) constructed using the molecular marker information, showed Praj 2.1.2 and Cowley still closer to each other as in the factorial analysis in Fig. 2. Most of the TS1 and Wray genotypes, both crosses and back crosses are centrally located on the tree. The sorghum genotypes that have been reported in previous studies to have high stalk-sugar content have been represented in red on the tree while those known to have lower stalk-sugar appear blue.

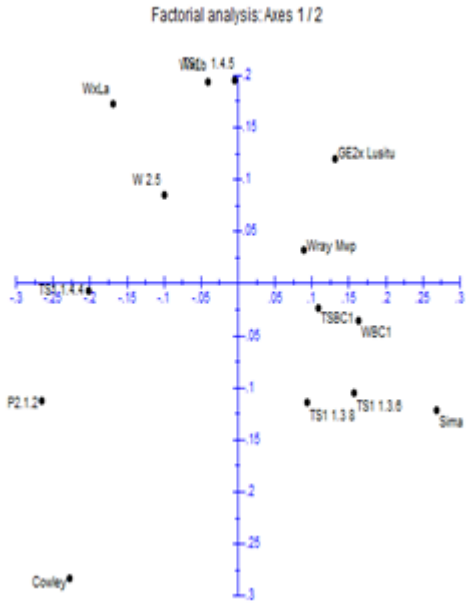

Fig. 2 Factorial analysis for the genotypes revealing their relative positions on two axes.

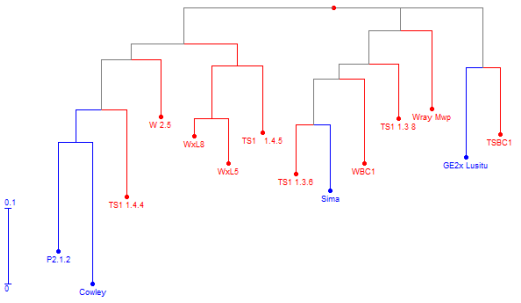

Fig. 3 Phylogenetic tree representation of the genotypes

TABLE 1
MARKERS AND THEIR POLYMORPHIC INFORMATION CONTENT (PIC).

Marker	Major allele frequency	Gene diversity	Heterozygosity	PIC
gpsb067	0.500	0.500	0.067	0.375
msbCIR238	0.500	0.612	0.000	0.541
SbAG02	0.938	0.117	0.000	0.110
xcup02	0.833	0.278	0.000	0.239
xcup16	1.000	0.000	0.000	0.000
xcup53	0.750	0.403	0.000	0.363
xcup61	0.824	0.304	0.000	0.281
xgap001	0.625	0.517	0.083	0.444
xgap342	1.000	0.000	0.000	0.000
xtxp014	1.000	0.000	0.000	0.000
xtxp015	1.000	0.000	0.000	0.000
xtxp034	0.576	0.600	0.231	0.551
xtxt040	0.722	0.444	0.000	0.409
xtxp141	0.308	0.734	0.000	0.684
xtxp145	0.357	0.783	0.786	0.755
xtxp176	0.675	0.439	0.450	0.342
xtxp298	0.625	0.570	0.000	0.539
xtxt312	0.000	1.000	NaN	1.000
xtxp357	0.618	0.472	0.059	0.361
xtxp012	0.454	0.645	0.000	0.579
Mean	0.665	0.421	0.083	0.379

IV. DISCUSSION

Considering that the twenty microsatellite markers in the study reasonably covered the entire sorghum genome (at least one marker to represent each of the ten sorghum chromosomes), the performance of the twenty markers on average was good. The mean PIC for all the markers across the genotypes in the study was 0.3788. Markers xtxp014, xtxp015, xtxp016 and xgap342 had a PIC value of 0.00 and were therefore monomorphic. This may mean that the sorghum genotypes in the study probably possess an identical allele combination at these loci. [8]

Two of the markers used in this study are known and reported in previous studies to be associated with the trait of high sugar in sweet sorghum [9], [10], [11]. These are xtxp014 and xtxp034. We found that the marker xtxp014 was highly monomorphic with only one allele while xtxp034 was quite polymorphic with four different alleles across the genotypes. Therefore, xtxp034 was more useful in the separation of genotypes based on their sugar levels. Of the twenty markers, one marker xtxp312 did not work at all. Reasons for this observation range from inaccessible target regions for amplification, to poor marker-genotype association. [12]

The rest of the markers clustered the sorghum genotypes meaningfully well, and jointly provide information on the genetic relatedness of the genotypes. In addition, the PIC and heterozygosity values of the markers provided insight into the usefulness of individual markers that can be included in future studies. Generally, the markers were sufficiently informative with regard to the diversity within the genotypes

The co-dominance nature of the microsatellite marker system made the organization and analysis of the data generated from the twenty markers quite manageable. However, in molecular marker application, plenty of phenotypic data is necessary for the smooth interpretation of results to give meaningful and helpful information applicable to crop research.

V. CONCLUSION

This study demonstrates that molecular marker application in crop science can yield vast amounts of information for breeding purposes. Variation within the sorghum gene pool is indeed sufficient for genetic improvement of several bioenergy traits, and molecular markers can be applied to facilitate the selection of these traits. Microsatellite markers are ideal for genetic assessment studies because they are co-dominant markers, are simple to use and comparatively less expensive even for countries with less advanced facilities for biotechnology research.

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APPENDIX 1

Source	d.f	B	PH	PG	MRSC	GW	PW	JV	STBMHA	TOTSUG
Line	17	39.637***	1.2525***	2.0455***	3.7694***	0.7014**	0.0165**	4827.3***	1.641E+09***	7.669E+11***
Rep	4	1.008	0.2057	0.3411	1.0236		0.0813	0.0040	1533.5	1.809E+09
Residual	68	8.054	0.1313	0.6018	0.4251		0.2534	0.0065	709.8	8.279E+08 1.268E+11
Total	89									

d.f: Degrees of freedom P <.001

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