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ANALYSIS OF GENETIC DIFFERENCE WITHIN AND BETWEEN OF WILD RELATIVES OF SORGHUM IN SUDAN, USING SSRs

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Abstract

Sorghum (*Sorghum bicolor* (L.) Moench) originated in East Africa specifically Sudan and Ethiopia where wild relatives of sorghum are widely distributed. The wild relatives harbored important genes. However they are often ignored and orphaned. This study aimed to explore genetic diversity among the accessions, and the results will enhance the utilization of wild sorghum in Sudan. 59 wild and cultivated sorghum accessions, representing three sorghum growing regions in Sudan: Gezira state (central), North Kordofan (Western), and the Gadareff area (Eastern). The materials were assessed for genetic diversity and population structure using a panel of 32 SSR markers. A total of 255 allelic variations were detected with a mean of 0.6721. The Polymorphic Information Content (PIC) value averaged 0.6301 showing the polymorphic and discriminatory nature of the selected markers. The populations showed little external gene-flow. Variations calculated with AMOVA are lower among populations (4%), moderate within individuals (23%), and higher among individuals within population (73%). A rooted neighbor-joining tree of four sorghum cultivars and 55 wild relatives revealed three distinct groups independent on their geographical origins. The results will be useful for future collection and utilization in genetics and breeding program.

Key words: Wild relatives, Sorghum, SSRS markers, Genetic diversity, Structure, Sudan.

Introduction

Sorghum (Sorghum bicolor L. Moench) originated in Sub-Saharan Africa, and is staple food crop in arid and semi-arid lands of the world (Balota *et al.,* 2008; Dillon *et al.,* 2007). The crop was first domesticated in Northeast Africa (Doggett, 1988). Taxonomically, sorghum has fifteen races: *bicolor*, *caudatum*, *kafir*, *durra* and *guinea* type, and10 intermediates (Harlan & de Wet, 1972). The crop is a highly diverse species belongs to the genus *Sorghum* of the tribe *Andropogoneae.*

Wild relatives of sorghum have two subspecies of *S. bicolor*: *S. bicolor Verticilliflorum* and *S. bicolor* subsp. *Drummondii* (Harlan and De Wet, 1972; Dogget, 1988). Wild relatives of sorghum are rich with genetic diversity and have a broad genetic base including novel and valuable traits like disease resistance (Gurney *et al.,* 2002; Kamala *et al.,* 2002; Reed *et al.,* 2002; Rao Kameswara *et al.,* 2003). Sudan is the one of Sub-Saharan African countries where wild sorghum populations were distributed in all sorghum cultivated areas, however little information is available, the wild relative of sorghum was not been surveyed.

Wild relatives of sorghum can provide different sources of resistance to biotic and a biotic stresses (Rich *et al.,* 2004). The varietal development requires plant genotypes harbor useful alleles of targeted genes to be used as donor parents. Wild accessions have long been tapped by plant breeders and yielded useful genes for their related cultivated species.

Identification and utilization of conserved genetic material of wild species helps to sustain crop improvement particularly in breeding for biotic and a biotic stresses. Information come out from genetic diversity studies permit the classification of genotypes into heterotic groups which are important for hybrid development and estimating the relative strengths of the factors affecting the genetic makeup as mutation, natural selection, migration, and genetic drift. Understanding of genetic variability is useful to create segregating populations with maximum genetic variability for further selection (Barrett & Kidwell, 1998). This information is also useful for better understanding evolutionary trends and will help in gene bank management and strategies for collection and conservation of the germplasm.

Genetic diversity has been extensively used worldwide to explore wild species using SSR technology. SSRs markers are widely used for DNA fingerprinting, linkage map construction and population genetic studies.

SSRs are unique PCR based markers due to their co-dominant nature which increases their efficiency for genetic mapping and population genetic studies.

Corresponding to different breeding uses and opportunities of wild relatives of sorghum, this study was undertaken to enhance utilization of wild relatives of sorghumin Sudan through; 1) assessment of genetic diversity and structure of wild relatives of sorghum, 2) detection of genetic relationships among individuals and populations, 3) providing essential information for breeding programs.

Materials and Methods

Germplasm: 55 wild relatives of sorghum entities were collected from three areas in Sudan: Central (Gezira), Eastern (Gadaref area), and Western (North Kordofan) which is irrigated, high and low rainfall areas, respectively (Fig. 1). Four cultivated sorghum cultivars were used as out groups.

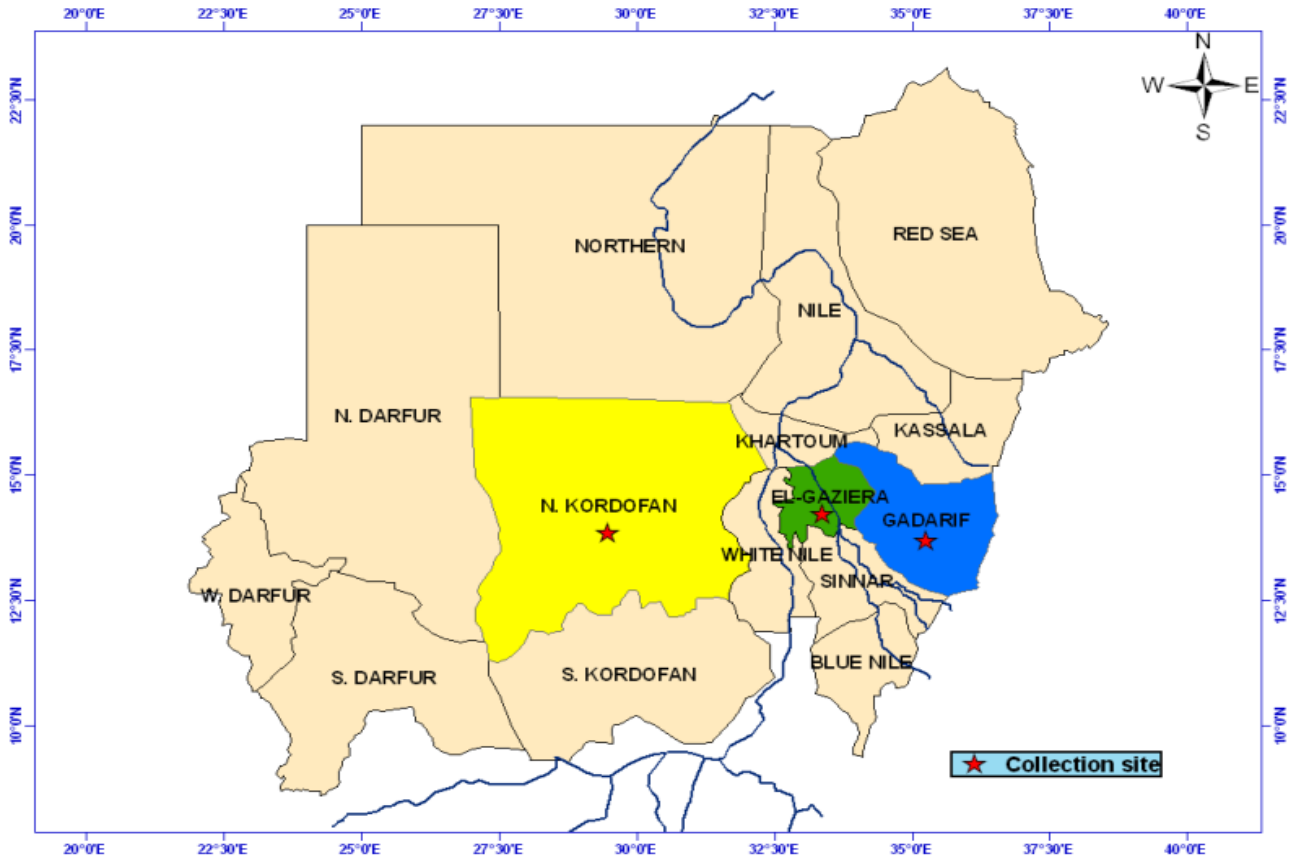

Fig. 1. Map showing the geographical regions of the collected 59 wild relative sorghum accessions and cultivated genotypes.

DNA extraction and marker analysis: Five to eight seeds from each of the 59 wild accessions and cultivated sorghum were planted in soil and incubated for 48 hrs to germinate in a greenhouse. Then, DNA was extracted from three to five fresh leaves collected when they were one week old and bulked. DNA was extracted from the samples using a modified CTAB extraction protocol as described by (Mace *et al.,* 2004). The genotypes were then assessed using 32 SSR markers covering all ten of sorghum's chromosomes using agrose gel electrophoresis for qualification and Nano-drop spectrophotometer (Billot *et al.,* 2012) for quantification.

Genotyping: The PCR conditions were optimized as follows: denaturation at 94°C for 30sec, followed by annealing at 56°C for 1min and extension at 72°C for 2min repeated 35 cycles, and finally extension at 72°C for 20min. Then, the PCR products were prepared in 386-well plates, with formamideHidi and GS500liz standard size, 1000µL to12Ml respectively, which 0.7 µ> of the amplified DNA co-loaded four florescent markers (Fam, Vic, Ned, and Pet), to 8µl from the mixture. Then the prepared 384 well plates were transferred to the sequencer. Scoring of peaks was done using Genemapper program (Applied biosystems, softwarev.4.0).

Data analysis: The genotyping data was manipulated and analyzed using allelobin (for allele standardization and determining the quality of the markers used, PowerMarker v3.25 (Liu & Muse, 2005) to show the performance of the markers and discriminatory ability, GenAlex 6.3b (Peakall & Smouse, 2012) for allelic pattern, genetic distance, similarity and AMOVA, DARwinv5 for cluster analysis and dendrogram, and structure v2.3.4 (Pritchard *et al.,* 2000).

Results and Discussions

Performance of SSRs markers: 32 SSRs markers were used to assess genetic diversity of 55 wild relatives using 4cultivated accessions as an out group (Table 1). The marker quality index had a mean of 0.32, ranging between 0.0005 for marker Xtxp136 to 0.5114 for marker Xcup53. The selected SSR markers revealed a total of 255 alleles among the 59 wild relatives and cultivated sorghum accessions (Table 1). The average number of alleles per SSR marker was 8, ranging from 3 alleles for markers Xcup61, Xtxp136 and Xiesp 0107, to 18 alleles for markers Xtxp 012 (Table 1). When we considered alleles with frequency ≥ 5% as common alleles, the number of detected alleles were reduced to 82(32%), the average will be reduced to 6.3, ranging from 3 alleles for markers Xcup 61 and Xiesp 0107 to 10 alleles for marker Xgap 72. Also, when we considered alleles with frequencies below 5% as rare alleles, a total of 173 (68%) rare alleles were detected (Table 1), with an average number of 9.1 per SSR marker, ranging from 3 alleles for marker Xtxp 136 to 18 alleles for marker Xtxp 012. Polymorphic information content (PIC) across 32 SSR markers had a mean of 0.6301, ranged from 0.3711 for marker Xiesp 0107 to 0.9074 for marker Xtxp 012 (Table 1).

Table 1. Summary of marker performance.

Marker	Major Allele frequency	Allele No	Availability	Gene diversity	Hetero-zygosity	PIC	Quality index
CIR286	0.56	7.00	0.88	0.57	0.12	0.50	0.33
gpsb123	0.39	5.00	0.88	0.72	0.08	0.67	0.00
CIR238	0.39	11.00	0.98	0.78	0.14	0.76	0.31
CIR246	0.38	7.00	0.93	0.73	0.16	0.69	0.32
CIR248	0.57	9.00	1.00	0.61	0.14	0.57	0.43
CIR300	0.42	6.00	0.97	0.70	0.39	0.65	0.09
CIR329	0.57	6.00	0.88	0.62	0.19	0.58	0.27
Sb5-206	0.36	12.00	0.92	0.80	0.20	0.78	0.50
Xcup02	0.40	6.00	0.95	0.71	0.11	0.66	0.19
Xcup61	0.66	3.00	0.88	0.47	0.10	0.39	0.18
Xcup63	0.46	4.00	0.95	0.61	0.21	0.54	0.21
Xgap72	0.55	10.00	0.95	0.64	0.20	0.61	0.42
Xgap84	0.41	9.00	0.86	0.71	0.16	0.67	0.28
Xgap206	0.30	15.00	0.90	0.85	0.42	0.84	0.44
Xiesp0107	0.66	3.00	0.83	0.46	0.39	0.37	0.33
Xtxp012	0.15	18.00	0.88	0.91	0.35	0.91	0.42
Xtxp021	0.32	11.00	0.83	0.82	0.24	0.80	0.50
Xtxp040	0.65	5.00	0.97	0.52	0.12	0.47	0.43
Xtxp136	0.38	3.00	0.95	0.66	0.63	0.59	0.00
Xtxp141	0.54	8.00	0.95	0.66	0.20	0.64	0.09
Xtxp145	0.43	13.00	1.00	0.78	0.07	0.76	0.38
Xtxp273	0.41	10.00	0.90	0.77	0.30	0.75	0.35
Xtxp278	0.43	5.00	0.88	0.69	0.21	0.64	0.32
Xtxp321	0.52	9.00	0.92	0.66	0.15	0.61	0.46
Xcup11	0.73	5.00	0.81	0.44	0.04	0.41	0.23
gpsb067	0.62	7.00	0.86	0.59	0.16	0.56	0.24
CIR306	0.72	7.00	0.85	0.46	0.08	0.43	0.50
Xcup14	0.55	3.00	0.86	0.54	0.18	0.45	0.19
Xcup53	0.42	8.00	0.83	0.70	0.16	0.65	0.51
Xtxp010	0.41	8.00	0.81	0.73	0.04	0.69	0.43
Xtxp320	0.23	12.00	0.81	0.86	0.15	0.84	0.49
CIR283	0.45	10.00	0.97	0.73	0.11	0.69	0.45
Mean	**0.47**	**7.97**	**0.90**	**0.67**	**0.19**	**0.63**	**0.32**

Comparison of genetic diversity between different growing regions: A total of 32 SSR markers covering the sorghum genome were optimized and used to separate 55 wild relatives of sorghum representing three agricultural regions(high and low rainfall and irrigated) in Sudan along with four cultivated lines as an internal control accessions. The Nei's unbiased estimate of genetic diversity (*H*) among the 59 wild relative accessions and cultivated varieties across the 32 loci was 0.6721. The allelic patterns and genetic diversity parameters across the different populations were calculated to determine the genetic differences between the 59 wild relatives and cultivated sorghum accessions. The number of common alleles were 7.3 (SE0.171), 20.5 (SE0.269), 21.8(SE3.5), and 3.59 detected in Central, Gadaref, Western, and Out-group, respectively (Table 2a). The number of different alleles detected was 3.9 (SE0.315), 5.656 (SE0.439), 2.344 (SE0.172), and 6.188 (SE0.40) in Central, Gadaref, Western, and Out-group, respectively (Table 2a). The mean number of effective alleles was 2.887 (SE0.126), while values of 3.049 (SE0.251), 3.316 (SE0.263), 1.979 (SE0.145), and 3.316 (SE0.266) were detected in Central, Gadaref, Western, and Out-group, respectively (Table 2a). The Shannon Index averaged 1.101 (0.044) with values 1.128 (SE0.0075), 1.281 (SE0.075), 0.638 (SE0.78), and 1.337 (SE0.071) detected in Central, Gadaref, Western, and Out-group, respectively (Table 2a). The observed heterozygosity had a mean of0.180 (SE0.014) which is lower than the unbiased expected heterozygosity 0.609 (SE0.019). While the observed heterozygosity was 0.179 (SE0.028) lower than the unbiased heterozygosity 0.658 (SE0.028) in Central, observed heterozygosity 0.209 (0.028) lower than the unbiased heterozygosity 0.651 (SE0.025) in Gadaref, observed heterozygosity was 0.146 (SE0.034) lower than the unbiased heterozygosity 0.465 (SE0.0.053) in the out-group, and observed heterozygosity was 0.186 (SE0.024) lower than the unbiased heterozygosity 0.663 (SE0.24) in Western (Table 2a). Genetic polymorphism was averaged to 94.5%, with values between 78% (25) and 100% (32) of 32 SSR markers used (Table 2b).

Table 2a. Parameters used to determine level of genetic diversity in the wild relatives of sorghum.

Pop		N	Na	Ne	I	Ho	He	uHe	F
C	Mean	7.313	3.906	3.049	1.128	0.179	0.612	0.658	0.728
	SE	0.171	0.315	0.251	0.075	0.028	0.027	0.028	0.039
G	Mean	20.500	5.656	3.203	1.281	0.209	0.635	0.651	0.670
	SE	0.269	0.439	0.263	0.075	0.028	0.025	0.025	0.043
Out	Mean	3.594	2.344	1.979	0.658	0.146	0.398	0.465	0.561
	SE	0.109	0.172	0.145	0.078	0.034	0.045	0.053	0.085
W	Mean	21.781	6.188	3.316	1.337	0.186	0.648	0.663	0.704
	SE	0.356	0.400	0.266	0.071	0.024	0.024	0.024	0.040
Total	Mean	13.297	4.523	2.887	1.101	0.180	0.573	0.609	0.672
	SE	0.717	0.218	0.126	0.044	0.014	0.018	0.019	0.027

Key: Na = No. of Different Alleles, Ne = No. of Effective Alleles = 1 / (Sum pi^2), I = Shannon's Information Index = -1* Sum (pi * Ln (pi)), Ho = Observed Heterozygosity = No. of Hets / N, He = Expected Heterozygosity = 1 - Sum pi^2, uHe = Unbiased Expected Heterozygosity = (2N / (2N-1)) * He, F = Fixation Index = (He - Ho) / He = 1 - (Ho / He).

Table 2b. Percentage of Polymorphic Loci.

Population	% P	No. polymorphic markers
Central	96.88%	31
Gadaref	100.00%	32
Out-group	78.13%	25
Western	100.00%	32
Mean	93.75%	30
SE	5.26%	

Table 4. Pairwise population matrix of nei genetic distance and identity.

	Central	Eastern	Out-group	Western
Central		0.21	0.513	0.249
Eastern	0.811		0.504	0.091
Out-group	0.599	0.604		0.48
Western	0.779	0.913	0.619	

Genetic relationship of accessions: Analysis of molecular variance (AMOVA) calculated low variance among populations (4%) and moderate variance within individuals (23%) and among individuals within a population (73%) (Table 3). The F-statistic indicates that low population differentiation (Fst=0.035, P=0.010), high inbreeding level (Fis=0.76, P=0.010), and deviation from Hardy-Weingberg equilibrium (Fit=0.76, P=0.010) (Table 3). Genetic identity based on Nei's results showed a low similarity between out-group and Central of 0.599 while a high similarity between Western and Eastern populations was determined as 0.913 (Table 4). The results of genetic distances based on Nei's showed low genetic distance between Western and Eastern population at 0.091 while a high genetic distance showed between out-group and Central of 0.513. A rooted neighbor-joining tree showed three distinct groups independent on their geographical regions (Fig. 2). The first group includes accessions from western and Eastern. However, the cultivated sorghum (out-group) was sub-grouped together with some accessions from Eastern, Western and Central. The third group includes accessions from Western, Central, and Eastern.

Identity Bayesian model-based cluster analysis for 59 wild and cultivated sorghum accessions assuming only two genetic groups (K=2) (Fig. 3). The results did not correspond with genetic structure and geographic origin.

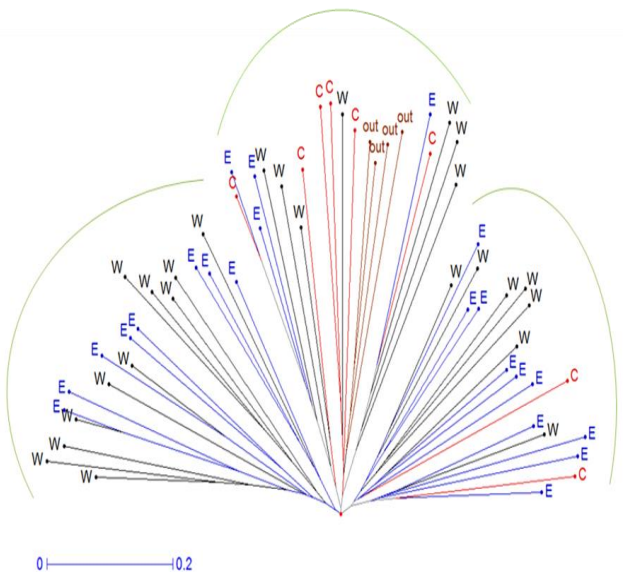

Fig. 2. Neighbor-joining tree of 59 wild relative sorghum accessions and cultivated genotypes based on the dissimilarity matrix of 32 SSR markers. Each color represents the different population groups (Central Sudan, *red*; Eastern Sudan, *blue*; Western Sudan, *black*; Out-group, *brown*).

Table 3. AMOVA and F-Statistics.

Source	df	SS	MS	Est. Var.	%	F-Statistics	Value	P (rand >= data)
Among Pops	3	92.3	30.8	0.4	3%	Fst	0.04	0.010
Among Indiv	55	1104.8	20.9	8.7	73%	Fis	0.76	0.010
Within Indiv	59	165.0	2.8	2.8	24%	Fit	0.76	0.010
Total	**117**	**1362.1**		**11.9**	**100%**			

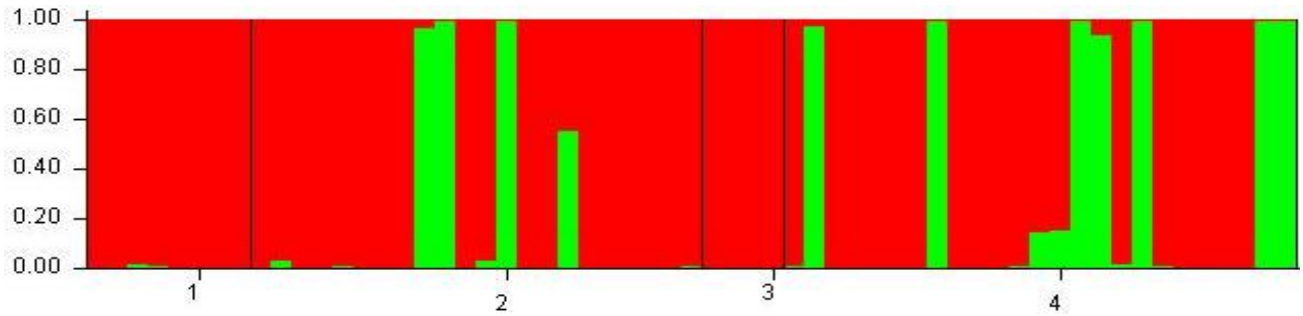

Fig. 3. Baysian model based structure (K=2) of 59 wild relatives and cultivated sorghum. Each accessions is represented by a single vertical line, each color represents one cluster as calculated by STRUCTURE. Key: 1=Central; 2=Eastern; 3=out-group; 4=Western.

Discussions

Thirty two SSRs markers covering the sorghum genome with at least two markers per chromosome were used to assess genetic diversity of 55 wild relatives and 4cultivated sorghum accessions. The selected SSR markers revealed a total of 255 alleles among the 59 wild relatives and cultivated sorghum accessions which is considered relatively high when compared to (Mutegi *et al.,* 2011) who detected 238 alleles in 110 wild accessions collected in Kenya. The average number of alleles per SSR marker was 8, this is also a high value when compared to 5.9 reported alleles per marker in elite sorghum lines and 100Guinea-race sorghum accessions (Smith *et al.,* 2000; Folkertsma *et al.,* 2005).

The Nei's unbiased genetic diversity (*H*) among the 59 wild relative accessions and cultivated varieties across the 32 loci estimated 0.6721 which is high compared to (Casa *et al.,* 2005) who examined 104 accessions comprising 73 landraces and 31 wild sorghums using 98 simple sequence repeat (SSR) loci distributed throughout the genome. The number of common alleles 7.3, was high compared to an average of 5.9 previously reported in elite sorghum lines (Smith *et al.,* 2000). The Shannon Index average was 1.101(SE0.044) showing a high level of genetic diversity. The mean observed heterozygosity was 0.180 (SE0.014) which is lower than the unbiased expected heterozygosity 0.609 (SE0.019) This result suggests that the populations are isolated and less exposed to external gene-flow, but in-fact the wild relatives of sorghum grown naturally as weed in sorghum field, therefore this argument make a suggestion of self-compatibility for wild sorghum plants. Self-compatibility was further suggested by low population differentiation (Fst=0.035, P=0.010) when the result showed high inbreeding level (Fit=0.756, P=0.010) and deviation from Hardy-Weinberg equilibrium (Fit=0.764, P=0.010). The three factors as Fst, Fis, Fit determine how the population maintained, and level of the inbreeding, drift and out-crossing. In Sudan, wild species grown naturally in sorghum cultivated areas, but it's very difficult to observe changes in wild populations. Several plant species are highly domesticated and their survival potential without human assistance is relatively low (Tesso *et al.,* 2008). The low gene-flow (Fst) value contrasts with (Ghebru *et al.,* 2002) who calculated high gene-flow (F_{ST}=0.50) value in 28 Eritrean sorghum accessions studied using 15 SSRs markers.

Analysis of molecular variance (AMOVA) showed low variance among populations (4%), moderate variance within individuals (24%), and high variance among individuals within population (73%).In Sudan, the seed supply system is highly dependent on an informal seed system along with a flow of seeds from the Eastern area, the center of origin of the crop leaving a high probability of wild seeds moving from the Eastern area to other parts of the country. However, the large differences detected among individuals within a population might be due to inbreeding and genetic drift.

The obtained genetic identity based on Nei's The results concur with our previous result when low differences between populations of sorghum germplasm collected from Sudan (Gamar *et al.,* 2013).

A rooted neighbor-joining tree showed three distinct groups independent of their geographical regions. The first group includes accessions from the Western and Eastern areas. However, the cultivated sorghum (out-group) was sub-grouped together with some accessions from all three regions. The third group included accessions from all areas. Similar results were obtained in sorghum by (Menz *et al.,* 2004), who found that sorghum lines were grouped according to their working groups.

Identity Bayesian model-based cluster analysis for the 59 wild and cultivated sorghum accessions assuming only two genetic groups (K=2), and the results did not correspond genetic structure and geographic origin. Structure analysis revealed consistent with previous molecular marker obtained when examining wild relatives (Ayana *et al.,* 2000a; Adugna *et al.,* 2013; Barnaud *et al.,* 2009), and have been reported in Africa for cultivated sorghum (Ayana *et al.,* 2000b; Nkongolo & Nsapato, 2003; Deu *et al.,* 2008).

Conclusion

The results revealed high allelic richness and genetic diversity in the wild relatives of sorghum in Sudan. Further, it showed genetic diversity among wild sorghum with low differences between populations. A rooted neighbor-joining tree developed using 32 SSRs markers separated 59 wild relatives and cultivated sorghum into three major clusters with no effect from their geographical origins. Identity Bayesian model-based cluster analysis assumed only two genetic groups (K=2). The study calculated high level of inbreeding and drift which means the wild types are isolated and/or were developed through aggressive selection in which is logically in consistent because the wild types are grown and maintained

naturally. These results suggest self-compatibility in wild relatives of sorghum which has not yet been explored and exploited. Wild relatives with useful traits, a broad genetic base and high diversity can be targeted in future studies using a molecular approach to restore lost genes and identify new sources of resistance.

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