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Genetic diversity study of improved cotton (*G. hirsutum* L.) varieties in Ethiopia using simple sequence repeats markers.

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In this work, the genetic diversity of 13 improved cotton (*G. hirsutum* L.) varieties which were released for production in Ethiopia were studied using 24 simple sequence repeats DNA markers. A total of 93 alleles, with a range of 2 to 9 and a mean of 3.88 per markers were detected. The polymorphism information content of the markers ranges from 0.13 to 0.80 with a mean of 0. 50. The pair-wise Dice genetic dissimilarity observed among the varieties were in a range of 0.16 to 0.56 and the overall mean was 0.39 indicating modest genetic diversity among released cotton varieties used in this study. Neighbor joining tree showed the varieties could be assigned to three clusters. Overall the result of this study alarms there is a need to introduce more diverse new cotton varieties to production to reduce the possible risk of genetic vulnerability.

Key words: Genetic diversity, *G. hirsutum* L., Simple sequence repeats

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INTRODUCTION

Cotton (*Gossypium spp.)* is often cross pollinated, world's most important fiber and major oil seed crop of global significance in the genus *Gossypium* L. of the family *Malvaceae*. It is native to tropical and subtropical climates, and is generally a shrubby plant having broad three-lobed leaves and seeds in capsules. The *Gossypium* genus comprises about 50 species, of which 45 are diploids (2n = 2x = 26) and five are allotetraploids (2n = 4x = 52) species (Fryxell, 1992; Wang *et al*., 2012; Yu *et al*., 2013). So far there are four cultivated species of *Gossypium* genus viz. *G. arboretum* L., *G. herbaceum* L., *G. hirsutum* L., and *G. barbadense* L. species (Ge and Wu, 2018; Noormohammadi *et al*., 2018; Shim *et al*., 2018).

Ethiopia has a long history in the cultivation and use of cotton since very ancient time (Nicholson, 1960; Gervers, 1990, 2008). However, it was not more than a home grown farm yard crop until the beginning of cotton improvement program in 1960's. Since then, production of old world cotton species by subsistence farmers for home use and to meet their family needs shifted to high yielding and better fiber quality possessing new world cotton (*G. hirsutum* L.) species on large scale commercial production for domestic industry needs and export. Furthermore, detailed cotton research plan and strategy including breeding, selection from foreign introductions, variety trials and other scientific studies resulted35 registered/released improved varieties (open pollinated, hybrids and genetically modified) of cotton for production (Gudeta *et al*., 2017). Hybrid and genetically modified

improved cotton varieties are yet to enter into production. On the other side, limited number of released open pollinated improved varieties are under large scale production and most are restricted to specific area of cotton growing belts of the country. However, they are being used as crossing parents to develop new improved cotton varieties.

Elucidating the details of genetic diversity in the available plant germplasm is one of the first step to understanding the conservation and the efficient utilization of plant germplasm for improvement purpose (Ulloa *et al*., 2006; Abdurakhmonov *et al*., 2012). Conventionally, genetic diversity in plant populations was determined by assessing differences based on visually observable morphological traits. Morphological traits(morphological markers) have economic merits of being readily available, simple and available for immediate use. However, they are subject to environmental variation, developmental stage of the plant and confounded by pleiotropic and epistatic effects (Govindaraj *et al*., 2015). In contrast, DNA markers exhibit superiority on handling some of these drawbacks of morphological markers and used as a tool for studying genetic diversity in various crop types including cotton (Messmer *et al*., 1993; Melchinger *et al*., 1993; Karp *et al*., 1996).

Since the dawn of DNA marker technology in the 1980s, a vast array of DNA-based genetic markers including simple sequence repeats (SSR) has been discovered. Simple sequence repeats are highly variable and evenly distributed throughout the genome. They are also common in eukaryotes and are ideal genetic markers for detecting differences between and within species of all eukaryotes (Farooq and Azam, 2002).

Simple sequence repeat DNA markers are highly polymorphic, robust, can be automated, requires very small DNA, highly abundant, analytically simple, readily transferable and have a co-dominant inheritance. Thus they are being widely applied in genetic diversity analysis and other scientific investigations (Powell *et al*., 1996; Matsuoka et *al*., 2002; Zhang *et al*., 2007; Ma et *al*., 2011). Hence this study was initiated with the objective of studying the genetic diversity among improved cotton (*G. hirsutum* L.) varieties released for production in Ethiopia using SSR markers.

MATERIALS AND METHODS

Plant Materials

In this study a total 13 cotton varieties were used. The seed source of all the varieties was national cotton research coordinating center, Werer Agricultural Research Center. The list of varieties used is given in Table 1.

SSR Markers

Initially, based on chromosome coverage, 46 SSR markers were selected from Cotton Marker Database of Cotton Gen (http://www.cottongen.org). The annealing temperatures of primers were optimized by performing gradient PCR (Altay Scientific S.P.A, Italy). Finally 24 polymorphic SSR markers were selected excluding 17 markers that failed to amplify properly and 5 that were monomorphic. Details of the SSR markers used for this study is in Table 2.

DNA Extraction

Seeds of the varieties were sown in plastic pots in greenhouse at Holeta National Agricultural Biotechnology Research Center, Ethiopia. Leaves were harvested from 12 days old seedlings and stored in freezer (-80^{O}C) to avoid degradation until DNA was extracted following, Xin *et al.* (2003). A small piece of leaf was collected in a 96 well PCR plate and 50µl of 0.1M NaOH and 2% Tween 20 (v/v) was added and incubated for 10 minutes at 95^{O}C using PCR machine. Subsequently 50µl of 0.1M Tris-HCl and 2mM EDTA was added and gently mixed at a moderate speed of 550 revolution per minute on vortex. A 0.5µl extract was used as a source of template DNA.

Polymerase Chain Reaction

The PCR was also performed according to Xin *et al*. (2003) with some modifications. The total volume was 10µl mixture, composed of 0.1% Bovine serum albumin (w/v), 0.25 µl each primer pairs [10µM], 0.5 µl crude DNA templates, 1% Polvinylpyrrolidone-40 (w/v), 2.9 µl Nuclease free water and 5 µl One Taq® 2X Master Mix with standard buffer (New England BioLabs®Inc.).

The thermal cycling conditions of the PCR was 2 minutes of initial DNA denaturation at 94^{0}C followed by 30 cycles consisted of 15 seconds of DNA denaturation at 94^{0}C 15 seconds of primer annealing at indicated temperature on Table 2 and 30 seconds of primer extension at 68^{0}C. The PCR cycling condition was terminated with final extension time of 5 minutes at 68^{0}C and the amplified product was hold at 4^{0}C.

Gel Electrophoresis

The amplified DNA was mixed with 6X loading dye with gel red in the ratio of 3:2 (v/v) respectively. With DNA ladder on the adjacent lane and the amplified products were loaded on 3.5% Hi-Res agarose gel in 0.5X TBE buffer (Xin *et al*., 2003; Wangari *et al*., 2013) and gels were run for 8 hour at 65V. Finally the amplified bands, were documented under ultraviolet light (Bio-Rad, USA).

Table 1. List of cotton varieties used in the study.

S. no	Variety name	Year of release	Designated code
1	STG-14	2014	G14
2	Werer-50	2015	G15
3	Candia	2014	G16
4	Claudia	2014	G17
5	Gloria	2014	G18
6	Weyto-07	2015	G19
7	Acala SJ2	1986	G20
8	Carolina queen	1994	G25
9	Sisikuk -02	2015	G26
10	Cucurova 1518	1994	G27
11	Deltapine-90	1989	G29
12	Ionia	2008	G31
13	Stam-59A	2007	G36

Table 2. SSR markers used in the study.

S. no	Name	Location	Motif (s)	Primers (5' - 3')	AT (^{o}C)
1	BNL2960	AD_Chr.10	GA	F- TAAGCTCTGGAGGCCAAAAA R- CCATTTCAATTTCAAGCATACG	52
2	BNL0946	AD_Chr.10, AD_Chr.20	GA	F- GCTGTTGCTCCACATCTCCT R- GGGCAAACAGATAGGCAGAA	58
3	JESPR0274	AD_Chr.9, AD_Chr.23, AD_Chr.26	CA	F- GCCCACTCTTTCTTCAACAC R-TGATGTCATGTGCCTTGC	57
4	BNL3545	AD_Chr.02, AD_Chr.14	CA	F- AGTCAGTTTTTTGTTAGCAATATGC R- AACCATTAATTCCCTATTTAACCG	52
5	DPL0196	AD_Chr.04, AD_Chr.22	GA	F- CACATTTGGTGGGTATTGAGAAG R- ACCATACACGTGCTAATGTCAAAG	56
6	BNL3594	AD_Chr.06, AD_Chr.25	TC	F- AGGGATTTTGATTGTTGTGC R-TGAATTCAAAACAAATGTTAGCC	51
7	NAU1119	AD_Chr.26	GCA	F- CCCCAACAAACTGAAAAATC R- AGTTCTGTTGCCTGGGTTAG	52
8	NAU3401	AD_Chr.12	ATC	F- ATGCCGACGCTTTAAGTAAC R- CGATATGGGCATGTTTGATA	52
9	NAU5189	AD_Chr.23	TTC	F- TGTCCCCCAATCATATTTTC R- CAACTTCCCAAGCTCGTATT	52
10	BNL4071	AD_Chr.05, AD_Chr.19	GT, GA	F- CATTTCAGAAGTTGACATTTTCG R- CACTGCCCCTAAGAAGTTGC	52
11	NAU1070	AD_Chr.03, AD_Chr.14	AGG	F- CCCTCCATAACCAAAAGTTG R- ACCAACAATGGTGACCTCTT	54
12	BNL3065	AD_Chr.16	AG	F- CAAACGGGAGACCAAAAAAA R- CGAACTGGCGAGTTAGTGCT	54
13	BNL3985	AD_Chr.23	TC	F- TTCAATTCTGGGTTCGAGCT R-CACCCATCAACCCAAATTTC	55
14	BNL1417	AD_Chr.25	AG	F- TTATTCTAACCACCGCCTCC R- TGAGTGGATATGCTTGGCCT	58

AT=Annealing temperature, F= Forward primer, R= Reverse primer.

Table 2. Continued.

S. no	Name	Location	Motif (s)	Primers (5' - 3')	AT (^{o}C)
15	BNL1721	AD_Chr.18	AG	F- TGTCGGAATCTTAAGACCGG	57
				R- GCGCAGATCCTCTTACCAAA	
16	BNL3371	AD_Chr.17	AG	F- CAATCCTTTACGTGGCCTGT	58
				R- AAAGACAGGCAATCCCCTTT	
17	BNL3644	AD_Chr.14	TC	F- GTGCTGTTTGGGCCTTACAT	58
				R- TAAGCGCATTGACACACACA	
18	DPL0717	AD_Chr.11,	TA,	F- CTCCATGATTTCTGAAACACAGGT	57
		AD_Chr.21	TTG	R- TTGTACTAACATACCTCCGGGTCT	
19	JESPR0220	AD_Chr.4	GA	F- CGAGGAAGAAATGAGGTTGG	56
		AD_Chr.22		R- CTAAGAACCAACATGTGAGACC	
20	BNL3992	AD_Chr.05,	TC,	F- CAGAAGAGGAGGAGGTGGAG	55
		AD_Chr.19	GA	R- TGCCAATGATGGAAAACTCA	
21	BNL1672	AD_Chr.09,	AG	F- TGGATTTGTCCCTCTGTGTG	57
		AD_Chr.23		R- AACCAACTTTTCCAACACCG	
22	BNL2634	AD_Chr.07,	AG	F- AACAACATTGAAAGTCGGGG	56
		AD_Chr.16		R- CCCAGCTGCTTATTGGTTTC	
23	NAU3665	AD_Chr.10,	AAT	F- CAGCATGGAAATCCTAATCC	54
		AD_Chr.20		R- TGAACTAGCTTGGCTGAATG	
24	NAU1297	AD_Chr.20	TTC	F- CCGCTGCAAAATTCTCTTAC	55
				R- CCCACTGGACATTTCTATCC	

AT=Annealing temperature, F= Forward primer, R= Reverse primer.

Data Scoring and Analysis

SSR profile within expected product size range was manually scored according to estimated molecular size comparing with DNA ladder and in the form of binary data (1 for presence and 0 for absent allele). Missed value was scored as -9. The allele number, allele frequency and polymorphic information content (PIC) of the SSR markers were calculated using Power Marker software program, version 3.25 (Liu and Muse, 2005).The PIC of SSR markers was calculated as per Botstein *et al*. (1980) using the following formula,

$$1 - \sum_{i=1}^{n} {p_i}^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2{p_i}^2{p_j}^2$$

Where, ${p_i}^2$ is the frequency of ithallele

Binary data was used to calculate the pairwise genetic dissimilarity among the varieties based on Dice dissimilarity (Dice, 1945) using DARwin software, version 6.0.18 (Perrier and Jacquemoud-Collet, 2006).

$D_{ij} = \frac{b+c}{2a+(b+c)}$

Where,

D_{ij} = Dice dissimilarity indices
a= number of alleles present in both i and j varieties
b= number of alleles present in i and absent in j
c= number of alleles absent in i and present in j
The pair-wise Dice dissimilarity matrix generated were used to cluster genotypes by Neighbor-joining algorithm (Saitou and Nei, 1987) using MEGA software, version 7.0.26 (Kumar *et al*., 2016).

RESULTS

Diversity among SSR Markers

A total of 93 SSR alleles were produced across 24 loci with a mean number of alleles 3.88 (Table 3). The detected number of alleles produced ranges from 2 to 9 and the lowest number of alleles produced belongs to each ofBNL2960, BNL3065, BNL1417, BNL1721 and DPL0717 locus while the highest number of alleles was detected in NAU1297 locus. The mean frequency of major allele of markers was 0.54 and within range of 0.27 to 0.92. The polymorphic information content (PIC), of the markers varied from 0.13 to 0.80 with a mean value of 0.50. The highest PIC values of 0.80 belongs toNAU1297 followed by 0.78 in BNL3545 and 0.73 in each of NAU1070 and

Table 3. SSR marker indices used in the study.

S. no	Marker name	Major allele frequency	Number of allele	Polymorphism information content
1	BNL2960	0.54	2	0.37
2	BNL946	0.77	3	0.34
3	JESPR274	0.38	3	0.59
4	BNL3545	0.27	6	0.78
5	DPL0196	0.38	7	0.73
6	BNL3594	0.81	3	0.29
7	NAU1119	0.64	3	0.47
8	NAU3401	0.42	3	0.57
9	NAU5189	0.48	4	0.45
10	BNL4071	0.54	3	0.54
11	NAU1070	0.35	6	0.73
12	BNL3065	0.88	2	0.18
13	BNL3985	0.42	5	0.67
14	BNL1417	0.92	2	0.13
15	BNL1721	0.92	2	0.13
16	BNL3371	0.52	5	0.62
17	BNL3644	0.46	4	0.60
18	DPL0717	0.54	2	0.37
19	JESPR220	0.50	3	0.50
20	BNL3992	0.54	3	0.50
21	BNL1672	0.42	5	0.66
22	BNL2634	0.54	4	0.45
23	NAU3665	0.50	4	0.55
24	NAU1297	0.27	9	0.80
Mean		**0.54**	**3.88**	**0.50**

Table 4. Dice dissimilarity matrix among cotton varieties.

Varieties	G14	G15	G16	G17	G18	G19	G20	G25	G26	G27	G29	G31	G36
G15	0.25												
G16	0.24	0.41											
G17	0.32	0.37	0.32										
G18	0.39	0.32	0.45	0.43									
G19	0.41	0.38	0.47	0.45	0.33								
G20	0.45	0.50	0.43	0.44	0.29	0.26							
G25	0.40	0.42	0.53	0.39	0.46	0.43	0.45						
G26	0.49	0.46	0.47	0.50	0.53	0.36	0.43	0.40					
G27	0.51	0.41	0.54	0.43	0.40	0.45	0.39	0.34	0.22				
G29	0.44	0.44	0.56	0.53	0.43	0.42	0.44	0.33	0.22	0.28			
G31	0.35	0.51	0.41	0.44	0.44	0.44	0.40	0.38	0.21	0.27	0.23		
G36	0.39	0.41	0.50	0.45	0.36	0.43	0.37	0.32	0.24	0.20	0.25	0.16	
Mean	**0.39**	**0.41**	**0.44**	**0.42**	**0.40**	**0.40**	**0.40**	**0.40**	**0.38**	**0.37**	**0.38**	**0.35**	**0.34**

DPL0196 while the lowest of 0.13 was recorded in each of BNL1417 and BNL1721.

Genetic Diversity among Cotton Varieties.

The Dice dissimilarity (genetic distance) matrix among *G.hirsutum* L. varieties used in this study is presented in Table 4. The overall mean genetic distance was 0.39. The pair-wise genetic distance ranged from low of 0.16 to high of 0.56. The highest pair-wise genetic distance observed was between, Candia (G16) and Deltapine-90 (G29) followed by Candia (G16) and Cucurova1518(G27), Carolina queen (G25), Gloria (G18) and Sisikuk-02(G26); and Claudia (G17)

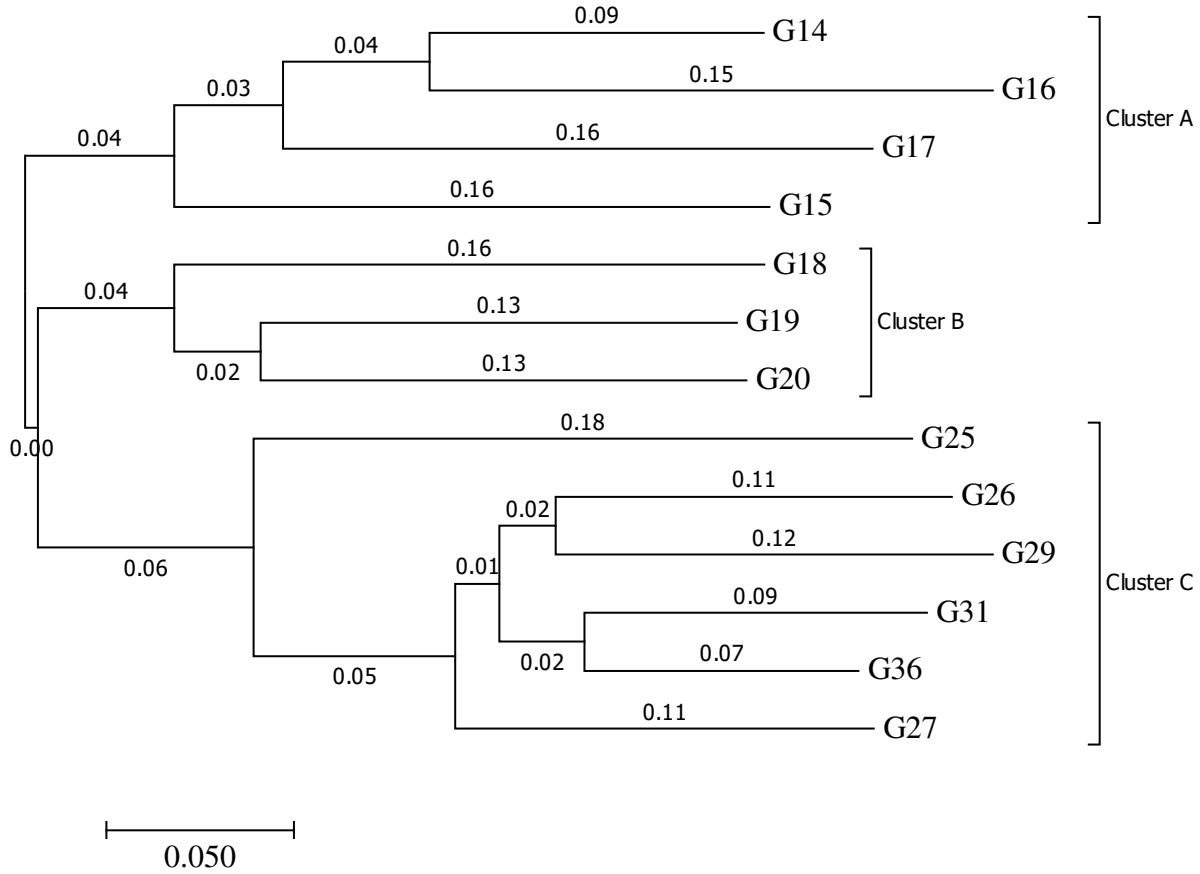

Figure 1. Neighbor- joining dendrogram showing genetic relationship of cotton varieties.

Table 5. Intra and inter cluster genetic distance among cotton varieties.

Cluster	A	B	C
A	0.32		
B	0.43	0.29	
C	0.46	0.42	0.27

and Deltapine-90 (G29). These varieties were initially introduced from breeding programs of different countries including USA and Turkey. The least genetic distance among the varieties was between Ionia (G31) and Stam-59A (G36), and they were selection from cotton germplasm introduced from Mali.

The Neighbor joining cluster analysis showed the varieties could be assigned to three clusters (A to C). Cluster A consists four varieties including STG-14 (G14), Werer-50 (G15), Candia (G16) and Claudia (G17). Cluster B comprises three varieties and they were Gloria (G18), Weyto-07 (G19) and Acala SJ2 (G20). Cluster C consists the highest number of varieties. Neighbor-joining dendrogram illustrating genetic relationship of studied cotton varieties is given in Figure 1.

The intra and inter cluster mean genetic distance among cotton varieties depicted in table 5. The mean intra genetic distance within varieties of each clusters were 0.32(Cluster A), 0.29 (Cluster B) and 0.27 (Cluster C). The lowest mean inter cluster genetic distance of 0.42wasamong varieties of cluster B and cluster C, followed by 0.43 (Cluster A and B) and 0.46 (Cluster A and C).

DISCUSSION

Understanding the range of genetic diversity in crop plants is critical for the effective conservation, management and efficient utilization. The present study is the first effort, SSR based genetic diversity study of improved cotton varieties in Ethiopia. The mean

frequency of major allele of markers was 0.54 and within range of 0.27 to 0.92, indicating that the 24 markers used were polymorphic in this study. The mean number of 3.88 alleles was generated per locus. A number of studies on *G. hirsutum* L. species cotton reported low mean number of alleles as compared to this study. For instance, a mean number of alleles of 2.13, with a range of 2 to 3 number of alleles per 31 SSR loci in 53 *G. hirsutum* L. cultivars reported by Bertini*et.al*, 2006. Similarly, Zhao *et al*. (2015) reported 2.26 per 146 SSR loci in 157 elite *G. hirsutum* L. cultivar collected from China, America, Africa, Former Soviet Union and Australia. In addition, Tyagi *et al*. (2014) reported a mean number of 3.06 (2 to 12) alleles in 378 upland cotton accessions per 120 SSR locus. In other studies comparatively high mean number of alleles reported. Zhang *et al*. (2011) reported 5.08 alleles per locus (2 to 14) in 59 cotton cultivars of China using 40 SSR markers. Lacape *et al*. (2007) reported a mean of 5.6 alleles per locus (2 to 17) evaluating 47 accessions of Gossypium genus through 320 SSR markers. Furthermore study by Moiana *et al*. (2012) on 35 cotton cultivars and eight inbred lines of *G. hirsutum* L. using 15 SSR markers showed a mean allele number of 6.9 (between 4 and 10) per locus. Overall, the number of alleles depends on markers to be used, platform used for resolution of amplified products and plant materials to be genotyped (Lacape *et al*., 2007).

The polymorphic information content (PIC), which is a measure of degree of informativeness of the markers varied from 0.13 to 0.80 with a mean value of 0.50. In this study, half (12) of the markers used were highly informative (i.e. PIC >0.50) and 9 (37.5%) markers were moderately informative with PIC values ranging between 0.29 and 0.50. The rest 3 (12.5%) markers were slightly informative with PIC values of 0.13 and 0.18. The mean PIC in this finding is more or less similar with that of Jia *et al*. (2014), who reported a mean PIC value of 0.53 in 325 *G. hirsutum* L. cotton germplasm using 106 SSR markers. In other studies, a mean PIC value of 0.36 (Moiana *et al*., 2015), 0.46 (Khan *et al*., 2009), 0.62 (Guang and Xiong-Ming, 2006), 0.80 (Zhang *et al*., 2011) were reported.

Genetic diversity among and within a given plant materials, is measured by genetic similarity (GS) or genetic distance (GD = 1 - GS), both of which indicate that there are either similarities or differences at the genetic level (Weir, 1990). The Dice overall mean genetic distance among the varieties used in this study was 0.39, indicating availability of modest genetic diversity among the varieties. The pair-wise genetic distance were in range of 0.16 to 0.56 indicating low to moderate genetic distances within the varieties. Nearly 59% of the observed pair-wise genetic distances were above the mean genetic distance (0.39). About 12.8% were within 0.50 and 0.56 range. Moreover, 16.6%, 23.1% and 46.2% of the pair-wise genetic distances were within range of 0.20 -0.29, 0.32-0.39 and 0.40-0.49 respectively. In relation to effective utilization of these varieties for future cotton improvement work, Candia (G16), Claudia (G17) and Werer-50 (G15) are on average the most promising to generate diverse segregates in which selection process could be effective.

The result of this study is in agreement with the work of, Guang and Xiong-Ming (2006) who reported a mean genetic distance of 0.39 among 43 sources of Upland cotton germplasm with different parental origins, breeding periods, and ecological growing areas in China using 36 SSR markers. In addition Bertini *et al*. (2006) reported a mean genetic distance of 0.41 (0.00 to 0.71) among 53 *G. hirsutum* L. cotton cultivars developed and released by public and private institutions in Brazil, Argentina and Paraguay using 31 SSR markers. Similarly, a mean genetic distance of 0.41(0.18 to 0.62) among 51 *G. hirsutum* L. cotton cultivars of different parental origins and breeding periods that were developed in Hubei Province in China using 108 SSR markers was reported by Tu *et al*. (2014).

The Neighbor-joining tree based on the estimates of genetic distance showed the varieties could be assigned to three clusters with the mean inter clusters genetic distance exceeding mean intra cluster genetic distance. Hence the crossing of varieties of different cluster is recommendable to generate diverse progeny. In Ethiopia, the tendency of cotton improvement efforts has been to select plant materials that combine the most desirable traits including high seed cotton yield and improved fiber qualities. The resulting homogeneity and uniformity offered substantial advantages in both the seed cotton yield and fiber qualities. However this promotes few and moderately distinct widely adapted varieties and enhances opportunities for pathogen or pest evolution and the natural selection of new strains able to attack their hosts successfully. Furthermore nowadays most of these varieties are under limited production areas. This, together with large-scale cultivation of limited number of varieties increase genetic vulnerability. In conclusion, there are promising varieties that can be exploitable as parental lines to generate segregating progenies of broad genetic base for further selection to develop improved population of genotypes which make up a cultivated variety. Overall there is a need to introduce more diverse new varieties to production to reduce the possible risk (genetic vulnerability).

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