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Genetic Purity Analysis of Maize (*Zea mays* L.) Hybrid Seed and Their Parents Produced in Different Seed Companies of Ethiopia

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Abstract. Genetic purity is one of the quality criteria required for successful seed production of maize. In hybrid seed production, genetic purity is contaminated due to out-crossing from other varieties or selfing events. In this study, Single nucleotide Polymorphisms (SNP) and Grow-out test (GOT) method was used for the objective to assess genetic purity of maize hybrid varieties with their parents produced by various seed growers in the country. Six three way cross hybrid (3WCH) maize varieties and seven single crosses were collected from different sources then planted with their parents in 2019. Genetic purity analysis by SNP revealed that 74% of an inbred line showed an acceptable genetic purity level (≥95%). However, five inbred lines (CML395, A7033, F7215, SC22 and 124-b(113)) revealed heterogeneity >0.05 ranged from 0.13 to 0.20. All single crosses except A7033/F7215 and all the 3WCH varieties showed the genetic purity level ranging from 40 to 66% and 44 to 63% across seed sources, respectively. Based on GOT results, the level of type in single crosses and 3WCH variety across seed sources ranged from 7.1 to 46.4% and 3.6 to 35.7%, respectively. Generally, in the current result both SNP and GOT showed both heterogeneity and homogeneity of seed. This implies the presence of variation among seed producers in terms of producing high quality seed. Therefore, awareness should have to be given for each individual seed producers on quality seed production techniques and procedures that they have to follow. Similarly, genetic purity analysis could be conducted further in the seed system to provide error correction and to ensure seed quality assurance and control.

Keywords: genetic purity; grow out test; hybrids; parent

INTRODUCTION

In maize production, different factors contribute to low yield. Seed quality is one of the most important limiting factors of the production potential of maize and it can be affected by both external and internal factors. Among different attributes of seed quality, genetic purity is the main feature that confers high quality to the seed. In maize, contamination can be occurring during hybrid seed production through self-pollination of female parents that can cause major problems on genetic purity of maize. Incomplete removal of tassel causes the major contamination that can increase the endogamic levels, reducing the genetic and physiological quality of the seeds that consequently decreases the crop productivity (Salgado et al., 2006). The best way to maintain genetic purity in maize seed hybrid production is isolation of the seed production fields, the removal of the female parental tassel, the cleaning of the harvest and processing machinery (Salgado et al., 2006). Seed quality is also judged by different end users, such as farmers, processors and industries. For instance, farmers expect to obtain high quality seeds that are able to germinate and produce normal seedlings under field conditions though in marginal land (Khan et al., 2012; Purba, 2020). High quality maize seeds have the capacity to produce vigorous seedlings across a wide range of environments in which low maize yields have been reported with the results of several factors, thereby information on reduction of seed quality parameters in maize inbred lines, and their heritability pattern are necessary for maize seed improvement (Adebisi et al., 2013).

Maize Production in Ethiopia has shown a considerable increment from 3.1 million tonnes in 2000 to 9.6 million tons (4.24 ton ha^{-1}) in 2019/2020, which represent production boosted by 209.7 % (CentralStatisticsAgency)., 2020). The

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reason for the increase of production and productivity of maize is development of improved variety. Over the last fifty years, Ethiopian Institute of Agricultural Research (EIAR) have released a number of different improved hybrid maize varieties for different maize growing agro-ecologies of Ethiopia. Among these, Bako national maize research center has contributed about 24 hybrid varieties suitable for mid altitude sub-humid agro-ecologies of Ethiopia (Bako National Maize Research Center, 2011).

Despite several maize hybrid varieties known for their improved yield performance, resistance to pests and diseases has been released, different factors affect maize productivity and production. Among these, seed quality is a key factor which influences crop productivity and production in the farmer's field and seed companies directly. Seed quality comprises genetic, physical, physiological quality (Sadjad, 1993), and pathology quality (Ilyas, 2012). Genetic purity is one of the main features required for successful seed production of maize hybrids. However, one of the major causes of genetic purity contamination in maize hybrid seed production is the self-pollination of the female parent which consequently decreases crop productivity (Salgado et al., 2006).

Different methods like molecular markers and grow out tests can be used for detecting genetic purity contamination of hybrid seed. Morphological markers are ineffective in large areas and environmentally influenced, and grow out test demand time and labor (Wani et al., 2017). Despite the grow out test taking time and labor it confirms results based on observation records on field. DNA based markers are an accurate and effective method in referring to contamination level and used to assess the genetic purity for a large number of species from a single seed of each material (Cooke, 1995).

Among different types of DNA markers available, single nucleotide polymorphism (SNP) marker are simple and the most suitable for genotyping of maize because of their low cost per data point, high genomic abundance, locus-specificity, simple documentation, and potential for high output analysis (Gowda et al., 2017). In addition to that, this marker can be used to assess the homogeneity of the inbred lines, the identity of the same inbred line/hybrid from different seed sources and parent-offspring relationship for single cross and three-way cross hybrids. Different authors Ertiro et al. (2015), Semagn et al. (2012), Ertiro et al. (2017), use this marker to detect genetic purity and identity of inbred line from different seed source and the genetic the level of heterogeneity (genetic variation) among maize inbred line. Currently, CIMMYT is also routinely using this technique and has identified a set of SNP markers which are used for routine quality control and quality assurance tests. Thus, it is crucial to assess the genetic purity of different commercial maize hybrids with their parents produced in different seed companies. Therefore, this experiment was conducted for the objective to assess the genetic purity of different maize hybrid varieties with their parental lines produced by various seed growers in the country and to determine the level of phenotypic off types by grow out test method (GOT).

METHODS

Sample preparation and Genotyping

The seed materials comprised of six maize (*Zea mays* L.) three way cross hybrids (3WCH) namely: BH660, BH661, BH546, BH547, AMH851 and AMH853 and seven single cross hybrids of the 3WCHs derived from eight different local seed companies indicated below in Table 1. Inbred lines for single cross formation and three-way cross formation (as a pollen parent) were also incorporated in the study. Seeds of the hybrids and their respective parental lines were planted in pots at Holetta Agricultural Biotechnology Research Center (HABRC) under greenhouse conditions. The experiment was laid out by three replications with completely randomized block design (CRD) in September 2019.

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Young and unbruised leaves samples collected at 4-5 leaves stages of the greenhouse grown maize plants, following the LGC protocol, were sent to CIMMYT-Kenya. The extracted DNA following the modified version of the CIMMYT high throughput mini-prep Cetyl Trimethyl Ammonium Bromide (CTAB) method (Semagn, 2014) as described elsewhere genotyped using the GBS platform at the Institute for Genomic Diversity, Cornell University, Ithaca, United States (Elshire et al., 2011), using 92 prescreened SNP markers that are regularly used by CIMMYT for maize seed quality control and assurance. Genetic purity analysis was calculated by the formula 1.

Table 1. Summary of Genetic materials and source of genetic materials

S.No	Hybrid variety	Source	Single Cross female parents	Source	Inbred lines	Source
1	BH660	C3	A7033/F7215	C1	A7033	GB
2	BH661	C1	A7033/F7215	C2	F7215	GB
3	BH661	C2	A7033/F7215	C4	142-1-e	TMSR(C1)
4	BH661	C6	CML395/CML2020	C1	CML395	TMSR(C1)
5	BH661	C7	CML395/CML2020	C2	CML202	TMSR(C1)
6	BH546	C1	CML395/CML2020	C3	SC22	GB
7	BH546	C3	CML395/CML2020	C4	124-b(113)	GB
8	BH546	C4	BKL002/CML312BK	C1	CML161	TMSR(C1)
9	BH547	C1	FS59/FS67	C8	CML161	C6
10	BH547	C3	Kit21/SRYSYN20	C8	CML165	TMSR(C1)
11	BH547	C4			CML165	C6
12	BH547	C5			BKL001	TMSR(C1)
13	Jibat	C8			BKL002	TMSR(C1)
14	Kolba	C8			CML312BK	TMSR(C1)
15	BH540	C4			BKL003	TMSR(C1)
16	BH540	C5			FS59	TMSR(C8)
17	BHQPY545	C6			FS67	TMSR(C8)
18	BHQPY545	C7			Kit23	TMSR(C8)
19					Kit21	TMSR(C8)
20					SRSYN20	TMSR(C8
21					F2-3SR	TMSR(C8)

C= Company, GB= Gene bank, TMSR= Technology multiplication and seed research

$$\text{Genetic purity (GP) (\%)} = 1 - \left[\frac{NH}{TSNP-MSNP}\right] x100 \quad \text{---------- (1)}$$

Where; NH = Number of heterozygous loci (different band nucleotide) of SNP
TSNP = Total number of single nucleotide polymorphism in the sample
MSNP = Number of missed single nucleotide polymorphism in the sample

Grow out Test Method

Grow out tests (morphological observation) were performed on the same seed lot sample left from molecular analysis. The total of six hybrid varieties and three single cross female parents were planted by using Randomized complete block design (RCBD) with four replications and four rows per plot. The plot was planted with two seeds

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per hill every 0.25m of 5.1m length and spacing 0.75m between each row. The purity of hybrid seed and their single cross parents conducted by GOT used the descriptor data that stringently related to seed quality. This was done by observing morphological characters based on the description of each hybrid varieties, especially color of anther, tassel, silk and overall appearance of the plant. While taking the observations, the plants showing deviations in descriptors against the standard check were tagged and examined carefully at a later stage to confirm whether they are off-types or not. The mean number of total plants and off-type plants from the experimental plot was recorded and converted to 400 plants since the minimum number of plants used for the GOT method is 400 plants. Finally, the result of the grow-out test was reported as the percentage of other off-type plants.

$$Percent\ (\%)\ of\ off\ type\ per\ plot\ = \frac{number\ of\ offtype\ per\ plot}{Total\ number\ of\ plants\ per\ plot} x100 \text{ ---------- (2)}$$

$$Percent\ (\%) of\ off\ types(400\ plants) = \frac{Mean\ number\ of\ off\ types\ (out\ 400\ plants)}{400} x100 \quad (3)$$

RESULTS AND DISCUSSION

Results

Genetic Purity (Homogeneity) test by molecular markers

The genetic purity (homogeneity) results of inbred lines, single crosses and three way cross hybrid varieties performed and indicated separately. The genetic purity level of inbred lines indicated in table 2. The results were variable across inbred lines, the homogeneity ranged from 80% to 100 % except CML161 collected from Company 6 (C6). Out of nineteen inbred lines, fourteen (74%) of inbred lines showed $\geq$ 95% genetic purity (homogeneity). However, five inbred lines revealed a homogeneity level between 80 and 93 %. In other words, they showed a high level of heterogeneity ranging from 7 to 20%.

Both molecular marker and growth test (morphological observation) methods distinguished the homogeneity level of single crosses and three way cross hybrid(3WCH) varieties across different sources.

The level of homogeneity (genetic purity) of seven single crosses collected from different seed sources indicated in figure 1. The overall single crosses showed homogeneity levels ranged between 40% and 81% across different seed sources. About 43% of single crosses showed genetic purity level between 50% and 59% at seven seed sources whereas 43% of single crosses showed between 60 and 66% of genetic purity at three seed sources. Based on the grow-out test method, the level off type in single cross parent and /or variety ranged from 2.1% (CML395/CML202) to 46.4% (BHQPY545) across seed sources (table 4).

The homogeneity level (genetic purity) of six three way cross hybrid (3WCH) varieties collected from different seed sources depicted in table 3. Based on the molecular result, the 3WCH variety showed the genetic purity level ranged from 44% to 63% with heterogeneity level ranged from 37 to 56% across different seed sources. Similarly, based on the grow-out test (morphological observation) the 3WCH variety expressed the off type level ranged from 3.6 % (BH546) to 35.7% (BH547) across seed sources (table 4).

Morphological observations (Grow out Test)

Grow out test results showed that the off type level in three way cross (3WCH) varieties ranged from 3.6% to 8.3% indicated in Table 4. The level of off type in 3WCH varieties was high at C3 and C4, which ranged from 20.2% to 27.4% and 28.6% to 35.7%, respectively. The single cross

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(CML395/CML202) female parent of BH546 and BH661 showed the level of off type ranged from 13.1% (C2) to 40.5% (C1) and 26.2% at both C3 and C4.

Table 2. Summary of genetic purity of different inbred lines at different seed source

S.N	Inbred line	Seed class	Seed source	Genetic purity/ Homogeneity (%)	Heterogeneity (%)
1	A7033	Nucleus	GB	82	18
2	F7215	Nucleus	GB	87	13
3	142-1-e	Prebasic	TMSR(C1)	95	5
4	CML395	Prebasic	TMSR(C1)	80	20
5	CML202	Prebasic	TMSR(C1)	100	0
6	SC22	Nucleus	GB	84	16
7	124-b(113)	Nucleus	GB	93	7
8	CML161(C1)	Prebasic	TMSR(C1)	98	2
9	CML161(C6)	Basic	C6	56	44
10	CML165(C1)	Prebasic	TMSR(C1)	98	2
11	CML165(C6)	Prebasic	C6	98	2
12	BKL001	Prebasic	TMSR(C1)	98	2
13	BKL002	Prebasic	TMSR(C1)	95	5
14	CML312BK	Prebasic	TMSR(C1)	98	2
15	BKL003	Prebasic	TMSR(C1)	95	5
16	FS59	Prebasic	TMSR(C8)	99	1
17	FS67	Prebasic	TMSR(C8)	98	2
18	Kit23	Prebasic	TMSR(C8)	98	2
19	Kit21	Prebasic	TMSR(C8)	97	3
20	SRSYN20	Prebasic	TMSR(C8	100	0
21	F2-3SR	Prebasic	TMSR(C8)	100	0

GB: Gene bank, TMSR: Technology multiplication and seed research, C1: Company 1, C6: Company 6

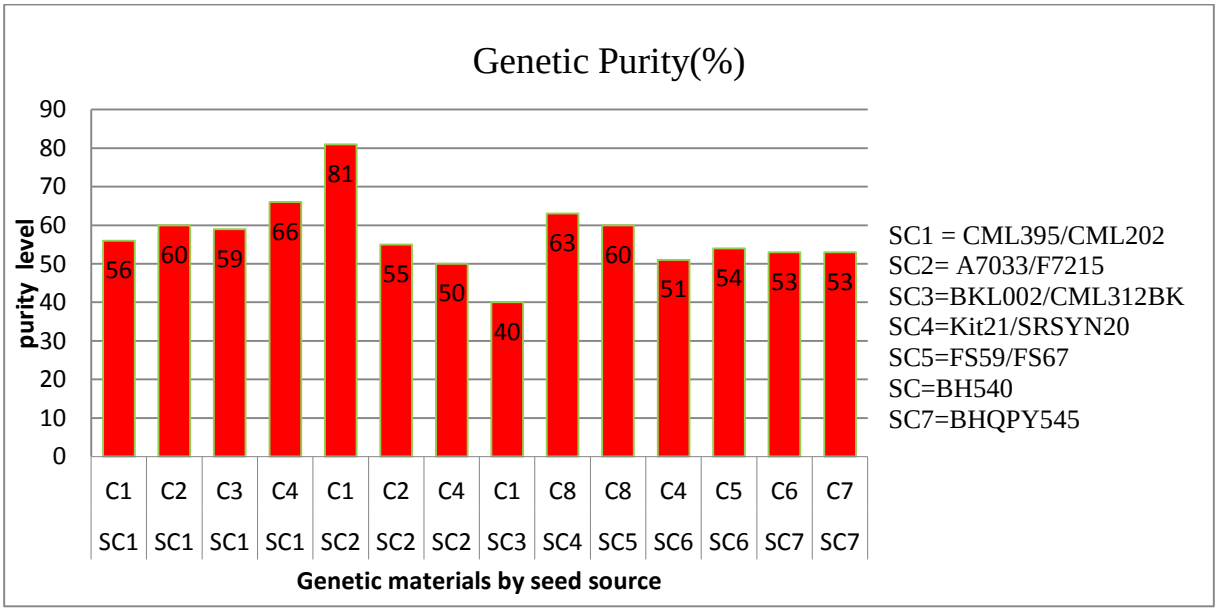

Figure 1. Summary of genetic purity of different single crosses at different local seed companies

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Table 3. Summary of genetic purity of different three way cross hybrid varieties at different local seed companies in Ethiopia

Three Cross hybrid variety	Seed Class	Seed Source (Companies)	Homogeneity/ Genetic purity (%)	Heterogeneity (%)
BH660	Certified	C3	54	46
BH661	Certified	C1	53	47
BH661	Certified	C2	45	55
BH661	Certified	C6	45	55
BH661	Certified	C7	44	56
BH546	Certified	C1	56	44
BH546	Certified	C3	63	37
BH546	Certified	C4	60	40
BH547	Certified	C1	53	47
BH547	Certified	C3	51	49
BH547	Certified	C4	50	50
BH547	Certified	C5	50	50
Kolba	Certified	C8	61	39
Jibat	Certified	C8	63	37

Table 4. The Purity (level of off type %) of hybrid seeds and their single cross parents at different seed companies based on Grow out test (GOT)

	Company1 (C1)		**Company2 (C2)**		**Company3 (C3)**		**Company4 (C4)**		**Company5 (C5)**		**Company7 (C7)**	
Variety Name	No plants (400)	% off type	No plants (400)	% off type	No plants (400)	% off type	No plants (400)	% off type	No plants (400)	% off type	No plants (400)	% off type
BH661	384.5	6.0	344.0	4.8	-	-	-	-	-	-	325.0	8.3
BH546	398.8	3.6	-	-	398.8	27.4	370.2	28.6	-	-	-	-
BH547	347.6	8.3	-	-	311.9	23.8	384.5	35.7	308.3	17.9	-	-
BH540	-	-	-	-	-	-	376.2	7.1	394.0	4.8	-	-
BHQPY545	-	-	-	-	-	-	-	-	-	-	320.2	46.4
BH660	-	-	-	-	304.8	20.2	-	-	-	-	-	-
CML395/CML202	344.0	2.1	379.8	13.1	361.9	26.2	384.5	26.2	-	-	-	-
BKL002/CML312BK	372.6	17.9	391.7	7.1	-	-	-	-	-	-	-	-
A7033/F7215	345.2	11.9	357.1	17.9	-	-	333.3	25.0	-	-	-	-

Discussion

Knowledge of genetic purity of seed is an essential component in the seed system for maintaining genetic identity of seed and ensuring seed quality through providing correction during seed production process. An inbred line to be considered as homogenous or genetically pure if the proportion of heterozygous loci for every SNP analyzed does not exceed 5% (Gowda et al., 2017; Semagn et al., 2012). In line to this, in our study,74% of an inbred line across the

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samples showed heterozygous loci less than 5% or the proportion of homogeneity greater than or equal to 95%. This indicated that these inbred lines are present at acceptable genetic purity level for seed production and absence of major contamination in the materials. Different authors Semagn et al. (2012), Ertiro et al. (2015), Prasad et al. (2019), Chen et al. (2016) and Ertiro et al. (2017) have also reported the acceptable level of genetic purity (homogeneity) among different sources maize inbred lines.

Any inbred lines within the samples showing the level of heterozygous loci between 5 and 15% could be required purification through performing ear-to-row selection where as one with >15% heterozygous loci is likely to be contaminated with unrelated genetic material and requires to be either discarded or extensive reselected for the original genotype (Gowda et al., 2017; Semagn et al., 2012). In the current study, five inbred lines namely A-7033 and F-7215 (Parents of BH660), SC22 and 124-b (113)) (parents of BH540) and CML395 showed the lower values below acceptable level for genetic purity. This results might be because of contamination occurred at initial time or out crossing occurred from foreign parent. The four former inbred lines or parents of both BH660 and BH540 also have early generation background for hybrid formation which may cause heterogeneity. This result harmony with those previously reported by Ertiro *et al* (2017) who found the heterogeneity value of inbred lines ranged from 12.5 to 31.5%. An inbred lines A7033 and F7215 which taken from the same seed source (C1) showed 82% and 87% homogeneity, respectively. Ertiro *et al* (2015) also reported the homogeneity level 76% and 84% for A-7033, 64% and 78% for F7215 through Comparison of Kompetitive Allele Specific PCR (KASP) and genotyping by sequencing (GBS), respectively. The parents of BH540 which are SC22 and 124-b(113) revealed homogeneity level 84% and 93%. Finding reported by Ertiro *et al* (2015) also showed the level of homogeneity 73% and 82% for SC22 and 89% for 124-b(113) by KASP and GBS, respectively.

Among single crosses, female parent CML395/CML202, which is common parent for high yielded commercial hybrid variety BH661 and BH546 showed homogeneity levels ranging from 56 to 66% at four different seed sources. Likewise, the grow-out test (GOT) method confirmed the genetic purity variation of CML395/CML202 across four different seed sources. The single cross (CML395/CML202) female parent of BH546 and BH661 showed the level of off type ranged from 2.1% (C1) to 26.2% (C3 and C4). Gemechu et al. (2020) also reported the presence of variation in genetic purity among different seed classes though molecular analysis and GOT method in Ethiopia. The single cross A7033/F7215, which was the female parent of hybrid variety BH660 showed homogeneity levels ranging from 50% to 81% across three different seed sources. Based on the (GOT), the off type level ranged from 11.9% to 25.0%. At company1, (C1) A7033/F7215 single cross showed homogeneity and heterogeneity level of 81% and 19%, respectively. This may indicate the contamination of single crosses through the selfing of female inbred line (A7033) during single cross formation at C1. Zivanovic et al. (2004) stated that homogeneity of heterozygous genotypes is increased due to an inbreeding of a given material or parent.

Based on the molecular results, the 3WCH varieties, BH661 and BH547 showed genetic purity level between 45% and 53% at three and four different seed sources, respectively. BH546 expressed genetic purity between 56 and 63%, at three different seed sources. Concerning to GOT results, BH546 and BH547 revealed the level of off type ranged from 3.6 to 28.6% and to 8.3 to 35.7%, respectively across different seed sources. In line to this result Gemechu et al. (2020) were reported the presence of off type across different seed sources for these hybrid varieties. In seed production, mechanical mixture is one the factors which cause

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genetic purity contamination which ultimately affect the performance of a variety and cause certified seed rejection. According to Gowda *et al*. (2017) in grow out test result if the off-type plants are more than 15 percent, the report should state that the sample consists of mixture of different genotypes. In line to this, some hybrid varieties and single crosses showed the level of off type greater than 15% at different seed companies which indicates the presence of physical mixture with others.

CONCLUSION

Genetic purity analysis through molecular analysis (SNP) and grow out test method (GOT) showed variation of purity level of different hybrid varieties and their parents across different seed sources. SNP revealed, 74% of parental inbred lines showed homogeneity (genetic purity) level of $\geq$ 95% whereas 26% (five inbred lines) showed heterogeneity level between 0.13 to 0.20. The presence of high heterogeneity level of these parental inbred lines may originate due to early generation of the lines. GOT showed 3.6 to 35.7% off type level in 3WCH varieties across different seed sources.

Generally, the current study showed the presence of heterogeneity of maize seed at parental lines (inbred lines) and hybrid seed production. In addition to this, variation was also observed among seed producers in terms of producing high quality seed. Therefore, it is recommended that further analysis could be conducted every season to ensure quality assurance and/ or quality control in the seed system, and seed producers also could give adequate attention to maize seed's genetic purity during their production process.

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