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Morpho-physiological Characteristics and Molecular Markers of Maize Crosses Under Multi-location Evaluation

Vasileios A. Greveniotis[1,2], Veronica S. Giourieva[3], Elisavet C. Bouloumpasi[4,5], Evangelia J. Sioki[6] & Paraskevi G. Mitlianga[4]

[1] Department of Agricultural Technology, Technological Education Institute of Thessaly, Larissa, Greece

[2] Department of Agricultural Development, Democritus University of Thrace, Orestiada, Greece

[3] Department of Botany, School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece

[4] Department of Agricultural Technologists, School of Agricultural Technology, Food Technology and Nutrition, Western Macedonia University of Applied Sciences, Florina, Greece

[5] Department of Food Science and Technology, School of Agriculture, Aristotle University of Thessaloniki, Thessaloniki, Greece

[6] Department of Agricultural Economics, Aristotle University of Thessaloniki, Thessaloniki, Greece

Correspondence: Vasileios A. Greveniotis. Department of Agricultural Technology, Technological Education Institute of Thessaly, Larissa, Greece. Tel: 30-241-068-4365. E-mail: vgreveni@mail.com; vgreveni@teilar.gr

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Abstract

The superior lines A1, A2, B1 and B2, which were developed from honeycomb evaluation in two different environments (A and B), were crossed in the field in environment A to obtain the following six crosses A1×A2, A1×B1, A1×B2, A2×B1, A2×B2 and B1×B2. Measurements performed in a RCB design included quantitative and quality characteristics and molecular data based on ISSR molecular markers. The objective of this study was to investigate the presence of variation of quantitative, quality and molecular characteristics among crosses of superior selected lines that developed after multi-location selection.

Principal component and cluster analyses were used for grouping genotypes, while correlations were performed to investigate relations between all quantitative and quality characteristics. According to PCA analysis, the six newly-developed crosses, which were evaluated in different environments, showed measurable distances between the identical lines (B1×B2 (A) and B1×B2 (B)) suggesting genotype-environment interaction. Also, cluster analysis showed that some crosses, such as A1×B2 (A) and A1×B2 (B), are grouped in separate and distinct clusters indicating that dissimilar developmental environments may cause changes in quantitative traits. This may be due to the origin of the selected lines, since they were developed in different locations. Lines developed in the same location gave crosses that had similar behavior in the two locations. Also, it is clear that a kind of gene fixation is apparent from the C3 cycle in A1xA2 cross, since it is close (and similar) to the C4 cross. Crosses including A2 line showed a greater stability in both environments.

Keywords: evaluation, ISSR, crosses, principal component analysis

1. Introduction

Maize (*Zea mays* L.) is one of the three most important crops worldwide, accounting for 40 percent of the world's cereal food production, because of its high adaptability in diverse environments (Koutsika-Sotiriou, 1999). It is cultivated in Greek regions of Macedonia, Thrace, Thessaly and West Hellas.

Plant breeding aims at crop yield potential and quality improvement by exploiting two types of vigor. One vigor is expressed in homozygous condition while the other in heterozygous condition (Fasoulas, 1988, pp. 16-18; Ipsilandis & Koutsika-Sotiriou, 2000). The term "inbred vigor" was introduced by Fasoulas (1973, 1974) to depict the superiority of recombinant lines over their original parental lines and also, the homozygote superiority over heterogeneity and crosses. Genome integration and genetic gain presupposes the fixation of desirable genes and the removal of deleterious genes (Ipsilandis & Koutsika-Sotiriou, 2000; Fasoula & Fasoula, 2005).

Maize kernel composition is important for human and animal nutrition (Flint-Garcia, Bodnar, & Scott, 2009). The kernel composition mean values for the commodity yellow dent corn on a dry matter basis are 71.7% starch, 9.5% protein, 4.3% oil, 1.4% ash, and 2.6% sugar (Watson, 2003). Kernel weight is an important component of maize yield in relation to genotypes across environments (Jones & Simmons, 1983; Borrás, Zinselmeier, Senior, Westgate, & Muszynski, 2009).

Characterization of maize breeding lines using simple sequence repeats (SSR) and inter-simple sequence repeat (ISSR) markers has been performed in the past (Žiarovska et al., 2013). The level of polymorphism reported ranged from 73 to 77% and the SSR polymorphism found among 45 maize genotypes reached 100%. Idris et al. (2012) recorded 69% polymorphism among maize genotypes based on the same molecular markers. Žiarovska et al. (2013) reported 6.8 markers per primer, while Warburton et al. (2001) reported 6.3 markers per primer. SSR and ISSR markers have been used in other species in order to identify and utilize genetic variation (Bredemeijer, Arens, Wouters, Visser, & Vosman, 1998; Gilbert, Lewis, Windson, & Caligari, 1999; Prevost & Wilkinson 1999; Huang & Sun 2000; Métais, Aubry, Hamon, Jalouzot, & Peltier, 2000; Prasad, Varshney, Roy, Balyan, & Gupta, 2000). PCR-based SSR markers are very powerful as they are co-dominant and multi-allelic, as well as highly polymorphic. However, SSR markers are costly and usually demand long time to be developed, which are major drawbacks for their use. In contrast, ISSR markers are universal, thus, there is no need for prior sequence knowledge and can be directly applied to any plant species. Thus, both systems have pros and cons, which means that there is no single dominant marker system suitable for universal use that meets all the user's needs, however ISSR markers are considered to be quick, robust and provide more informative data sets with less effort and cost than other dominant molecular marker techniques (Salimath, de Oliveira, Godwin, & Bennetzen, 1995; Yang, de Oliveira, Godwin, Schertz, & Bennetzen, 1996; Godwin, Aitken, & Smith, 1997).

Principal component analysis and cluster analysis are the most used multivariate techniques for morphological grouping of genotypes (Peeters & Martinelli, 1989; Mohammadi & Prasanna, 2003). With regard to maize, Khodarahmpour (2012) found three main clusters of maize hybrids based on 30 quality and quantitative traits.

The objective of this study was to investigate the presence of variation of quantitative, quality and molecular characteristics among crosses of superior selected lines that developed after multi-location selection.

2. Materials and Methods

2.1 Plant Material, Selection and Evaluation Methods

Selection started in 2007 in the F2 generation (C0) of the commercial F1 hybrid Costanza and continued for 5 cycles (up to C4). Selection was applied in two agro-climatically contrasting environments, environment A (Florina, Northwestern Greece, altitude 705 m, soil classification SL, soil pH = 6.25) and B (Trikala, Central Greece, altitude 120 m, soil classification SCL, pH = 8). Superior plants and lines (A and B according to the environment) were selected on the basis of the two selection equations (Fasoula, 2006, 2013).

In 2012, the best two lines A1 and A2 were selected after the 5-year evaluation (2007-2011) in environment A and the best two lines B1 and B2 were also selected in environment B. These superior lines A1, A2, B1, and B2 were crossed in the field in environment A to obtain the following six crosses A1×A2, A1×B1, A1×B2, A2×B1, A2×B2 and B1×B2. Rows were spaced 1 m apart and the usual crossing procedure was followed. The crosses were performed in August 2012.

In 2013, the six newly-developed crosses (A1×A2, A1×B1, A1×B2, A2×B1, A2×B2 and B1×B2) along with $_{F1}$ hybrid Costanza were sown in randomized complete block (RCB) trials in both environments A and B (Table 1). For environment A, an additional check material accompanied Costanza: a cross formed and selected from the previous generation of the two best experimental lines in environment A (A1×A2 in C3 cycle). Row spacing was 0.75 m.

Table 1. The genetic materials used in experiments in the two environments

1	Environment B	A1×A2
2	Environment B	A1×B1
3	Environment B	A1×B2
4	Environment B	A2×B1
5	Environment B	B1×B2
6	Environment B	A2×B2
7	Environment B	F1 Costanza
8	Environment A	A1×A2
9	Environment A	A1×B1
10	Environment A	A1×B2
11	Environment A	A2×B1
12	Environment A	B1×B2
13	Environment A	A2×B2
14	Environment A	F1 Costanza
15	Environment A	A1×A2 (C3)

2.2 Measurements Procedure

In 2014, measurements of quantitative and quality characteristics were conducted.

2.2.1 Quality Characteristics

Quality characteristics measured were: seed protein, seed oil, moisture, pH, ash content and color parameters. Milling of kernel samples was performed prior to the analyses.

Ash content was determined according to AACC Method 08-01 (AACC, 1983a). Moisture content was determined according to AACC Method 44-15A (AACC, 1983b). Total nitrogen was determined by a Kjeldahl method (modified AACC Method 46-12 (AACC, 1983c)) and the factor used was 6.25 for the calculation of protein content. Crude fat was determined by Soxhlet extraction with petroleum ether using a Soxtherm SOX 416 Macro (Gerhardt) based on AACC Method 30-25 (AACC, 1983d). The pH values were determined at 20 ^{o}C.

Color parameters (L*: luminosity, a*: redness and b*: yellowness) were measured using a Minolta Chroma Meter (model CR-410) (Minolta Camera Co, Osaka, Japan) with a 10 mm measuring area (aperture) and illuminant source C.

2.2.2 Quantitative Characteristics

Kernel weight: This was evaluated by counting and weighing 1000 maize grains (thousand-kernel weight). The weight of kernels per 100 litres was also calculated (hectoliter weight).

Kernel size: This was measured in mm by randomly selecting 10 kernels and measuring the three major axes, namely: length, width and depth with a Vernier Calliper.

Ear length, Ear diameter, Spindle diameter: The dimensions were measured in mm by the ruler and the calliper, as a mean of 10 ear observation for each genetic material.

Number of grain rows per ear: Number of rows was counted by eye, as a mean of 10 ear observation for each genetic material.

2.2.3 Molecular Analysis With ISSR Markers

The genetic diversity among the maize lines was studied using ISSR markers. Genomic DNA was isolated using the procedure described by J. J. Doyle and J. L. Doyle (1987) and was quantified using UV-Spectrophotometer Ultrospec 2000 (Pharmacia Biotech, Cambridge, UK). Samples were diluted to 20 ng/uL final concentration. Polymerase chain reaction (PCR) for ISSR analysis was performed in a total volume 20 uL. Each tube contained 20 ng DNA, 5 U/uL DNA *Taq* polymerase (Invitrogen, Carlsbad, CA), 1× PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 0.2 uM of each primer.

Six oligonucleotide primers complementary to simple sequence repeats were used for the study of inter-simple sequence repeats (UBC807, UBC811, UBC824, UBC827, UBC834, UBC841). The used primers are shown in Table 2. PCR amplification was performed in Veriti 96 canals (Applied Biosystems, CA, USA) as follows: an initial denaturation at 95 ^{o}C for 5 min, followed by 40 cycles of 30 s at 95 ^{o}C for denaturation, 1 min and 30 s at

54-58 ^{o}C (depending on the primer used) for annealing and 1 min and 30 s at 72 ^{o}C for elongation. Final extension was performed in 5 min step at 72 ^{o}C. PCR products were separated by electrophoresis in 1.5% w/v agarose gel and stained with ethidium bromide.

Table 2. ISSR primers used in this study

Primer name	Sequence (5'-3')
UBC834	AGAGAGAGAGAGAGAGT
UBC824	GAGAGAGAGAGAGAGAC
UBC841	ACACACACACACACACACT
UBC807	ACACACACACACACACG
UBC811	AGAGAGAGAGAGAGAGYT
UBC827	GAGAGAGAGAGAGAGAYC

To check the genetic diversity among the maize lines, which were grown in different environments, the bands of each primer were scored in an Excel 2013 file. Because ISSR markers are dominant, each band represents a phenotype at a single biallelic locus. The presence of a band was marked as 1 and the absence as 0 while the absence of an amplicon was marked as -1. A binary qualitative data matrix was formed. The data matrix was used to obtain the Principal of Coordinates Analysis (PCoA) using GenAlEx6.5 (Genetic Analysis in Excel, Peakall & Smouse 2012). Gene diversity and Shannon's diversity index (I) was used as a measure of within-population variability.

The aim of Principal Component Analysis (PCA) use is to establish the number of main factors that could be used in order to decrease the necessary numbers of effective parameters for the discrimination of genotypes. Moreover, this method could allow the correlation of phenotypic traits with genetic linkage between loci controlling traits (Iezzoni & Pritts 1991; Rakonjac, Aksic, Nikolic, Milatovic, & Colic, 2010).

Cluster analysis was used in order to separate the available data into groups of increasing dissimilarity. The Euclidean distance was used as a metric to measure the genetic dissimilarity of the 15 maize crosses, based on the combined quantitative and quality data and Ward's method was used for the agglomeration. Correlations were based on the procedure described by Steel and Torrie (1980), involving all quantitative and quality characteristics. The Pearson coefficient was used to measure the correlation between all quantitative and quality traits.

3. Results and Discussion

3.1 Qualitative and Quantitative Multivariate Analyses

Seventeen quality and quantitative traits were measured and the descriptive statistics of minima, maxima, means and standard deviations were recorded (Table 3). The results indicate morpho-physiological diversity. Some quality and quantitative traits display high coefficient of variation (CV) values: seed oil (18.92), ash content (11.75) and spindle diameter (10.63). The means of quality and quantitative were: for moisture 12.230%, for seed oil 2.96%, for ash 1.243%, for seed protein 8.498%, pH 6.5, L* 84.134, a* -0.909, b* 23.089, ear length 201.27 mm, ear diameter 49.28 mm, number of grain rows per ear 15.387, spindle diameter 26.811 mm, seed length 12.649 mm, seed width 8.883 mm, seed thickness 4.664 mm, thousand-kernel weight 356.192 g and hectolitre weight 70.519 kg·l^{-1}. Nuss and Tanumihardjo (2010) reported a typical kernel composition of: 9.5% protein, 4.5% oil, 1.5% ash, a little higher in comparison to our findings, even though kernel moisture content is a characteristic that depends on the environmental conditions at harvest with usually high CV (Jaradat & Goldstein, 2013). In our dataset CV for moisture content was relative low, indicating proper and homogeneous drying at harvest. Khodarahmpour (2012), reported 24.7% moisture, 30 grains per row, 14 rows per ear, ear diameter 4.1 cm, spindle diameter 2.4 cm, grain width 0.44 cm, grain diameter 0.68 cm, thousand-kernel weight 334 g and hectolitre weight 701.4 gl^{-1}.

Many references showed that genetic improvement of single-cross hybrids has been associated with increased tolerance to various biotic and abiotic stress factors (Duvick & Cassman, 1999; Tollenaar, Ying, & Duvick, 2000; Tollenaar & Lee, 2002; Duvick, 2005). According to Fasoulas (1993, pp. 111-114), high CV values indicate such behavior, *i.e.* increased influence of the environment. Seed oil and ash content with increased CVs are subjected to high environmental pressures.

According to Pearson coefficient, some traits showed a strong linear correlation (Table 4). The highest significant positive correlation was between hectolitre weight and b* (0.896), spindle diameter and ear diameter (0.859), and number of grains rows per ear and ear diameter (0.730). On the other hand, there were also high, significant, negative correlations like: L* and ash content (-0.627) and number of grain rows per ear and seed width (-0.634). Kernel colour usually exhibits strong relationship with other components of kernel (Kaur, Singh, & Rana, 2010; Jaradat & Goldstein, 2013). Rahman, Mukul, Quddus, Hassan, & Haque (2015) found no significant correlations between quantitative traits except for ear length with ear diameter.

According to the dendrogram (Figure 1), based on the quantitative traits, hybrids are differentiated into three main clusters. Some crosses, such as A1×B2 (A) and A1×B2 (B) are grouped in different and distinct clusters indicating that different developmental environments may cause changes in quantitative traits. This may be due to the different origin of the selected lines, since they were developed in different locations. Lines developed in the same location, gave crosses that had similar behavior in the two locations (like A1×A2). Also, it is clear that a kind of gene fixation is apparent from the C3 cycle in A1×A2 cross since it is close (and similar) to the C4 cross. On the opposite, crosses between lines of different locations showed greater distances in the two different locations, as was also reported by Shehzad, Okuizumi, Kawase, and Okuno (2009) and Ezzat, Ali and Mahmoud (2010) for sorghum. Crosses, including A2 line, showed a greater stability in the two areas. Additionally, it is also possible that increased stability of some crosses may be due to accumulation of favorable additive genes because of the certain breeding method of line selection (Fasoulas, 1988, pp. 54-56; Ipsilandis & Koutsika-Sotiriou, 2000). Khodarahmpour (2012), found three main clusters of maize hybrids, based on 30 qualitative and quantitative traits. The first one included most of the hybrids. Our findings from cluster analysis were confirmed by PCA analysis of quantitative traits (Figure 2). The plot grouped the crosses according to their phenotypic resemblance and morphological characteristics. The first two axis of PCA explained 49.58% of the total variation among the 15 crosses, while the first axis explained the 29.43% of the variation. The distribution of crosses represents the phenotypic variation existing among the crosses and also shows how extensively spread they are along both axes. There was found a significant grouping of phenotypic traits which contribute to seed yield and seed quality, characteristics which might be helpful for plant breeding. For instance, to generate high yield and superior quality lines, cross combinations could be performed between crosses with high ear diameter, spindle diameter, hectolitre weight and low pH. F1 commercial maize hybrid Costanza was grouped alone. The most significant traits of our datasets were revealed by PCA analysis, which allows the multivariate statistical analysis. Using Kaiser's criterion ("Eigenvalue" > 1) (Kaiser, 1958), the dimension implied by the 17 quantitative and qualitative traits to six components that explained the 89.155% of the total variation was reduced (Figure 3 and Table 5). Hafiz, Jehanzeb, Ejaz-Ul-Hasan Tahira and Tariq (2015) reported that principal component (PC) analysis showed that the first 4 PC factors had Eigen value > 1, explaining satisfactory from 86.7% and 88.4% of the total variation. Using cluster analysis, they classified 40 maize accessions into four divergent groups.

Table 3. Quality and quantity calculations of 17 characteristics of maize crosses

Variable	Minimum	Maximum	Mean	Std. deviation	CV (%)
Moisture	11.720	12.970	12.230	0.439	3.59
Seed oil	2.172	4.477	2.960	0.560	18.92
Ash content	1.062	1.464	1.243	0.146	11.75
Seed protein	7.413	9.884	8.498	0.616	7.25
pH.	6.330	6.720	6.507	0.113	1.74
L*	78.910	86.320	84.134		
a*	-1.810	0.200	-0.909		
b*	15.360	28.820	23.089		
Ear length	180.00	238.00	201.27	1.556	7.73
Ear diameter	42.445	52.590	49.280	3.085	6.26
Number of grain rows per ear	13.000	18.000	15.387	1.150	7.47
Spindle diameter	20.750	30.168	26.811	2.851	10.63
Seed length	11.750	14.240	12.649	0.867	6.85
Seed width	8.130	9.450	8.883	0.324	3.65
Seed thickness	4.230	5.000	4.664	0.216	4.63
Thousand-kernel weight	302.260	403.990	356.192	32.398	9.10
Hectoliter weight	63.100	75.140	70.519	3.272	4.64

Table 4. Correlation coefficients between 17 quantitative and quality traits

Variables	Moisture	Seed oil	Ash content	Seed protein	pH	L*	a*	b*	Ear length	Ear diameter	Number of grain rows per year	Spindle diameter	Seed length	Seed width	Seed thickness	Thousand-Kernel weight	Hectoliter weight
Moisture	**1**																
Seed oil	-0.091	**1**															
Ash content	-0.008	0.027	**1**														
Seed protein	-0.456	0.349	0.495	**1**													
pH	0.078	0.425	-0.229	-0.159	**1**												
L*	0.311	-0.213	**-0.627**	-0.448	0.171	**1**											
a*	0.15	0.406	**0.631**	0.28	0.225	-0.484	**1**										
b*	-0.508	**0.532**	**-0.528**	0.149	0.425	0.197	-0.24	**1**									
Ear length	0.047	**0.614**	0.261	0.217	-0.051	-0.465	**0.557**	0.188	**1**								
Ear diameter	0.339	-0.127	0.201	0.211	-0.42	-0.265	0.12	-0.316	0.349	**1**							
Number of grain rows per ear	0.295	-0.507	0.25	0.195	**-0.558**	-0.017	-0.146	-0.459	-0.182	**0.73**	**1**						
Spindle diameter	**0.549**	-0.073	0.38	0.002	-0.227	-0.403	0.297	**-0.517**	0.417	**0.859**	**0.565**	**1**					
Seed length	-0.25	0.065	**-0.579**	0.093	-0.051	0.371	-0.421	**0.537**	0.047	0.328	0.095	-0.071	**1**				
Seed width	-0.383	0.266	-0.227	0.014	0.442	-0.078	0.136	0.494	0.342	-0.218	**-0.634**	-0.219	0.308	**1**			
Seed thickness	0.16	0.183	-0.128	-0.192	0.327	-0.04	0.344	0.046	-0.037	-0.292	-0.314	-0.19	-0.42	-0.021	**1**		
Thousand-Kernel weight	-0.023	0.379	-0.312	0.236	0.318	0.161	0.226	0.486	0.208	0.304	-0.003	0.013	**0.563**	0.207	0.328	**1**	
Hectoliter weight	-0.492	0.475	-0.481	0.049	0.332	0.165	-0.03	**0.896**	0.289	-0.228	-0.463	-0.466	**0.579**	**0.528**	0.16	**0.611**	**1**

Values in bold are different from 0 with a significance level $\alpha = 0.05$.

Table 5. First six components from the PCA analysis of 17 quantitative and quality traits

	F1	F2	F3	F4	F5	F6
Moisture	4.511	0.437	0.137	31.685	1.047	7.784
Seed oil	4.837	12.567	0.087	0.967	0.043	19.982
Ash content	7.274	9.645	2.686	4.921	0.708	2.816
Seed protein	0.019	9.228	2.318	13.675	15.444	8.678
pH.	6.414	0.454	4.595	8.173	0.029	10.994
L*	1.831	15.237	0.618	6.038	0.376	12.471
a*	0.407	18.970	3.743	2.804	0.988	0.084
b*	16.156	0.203	2.352	0.437	0.192	0.971
Ear length	0.092	18.824	1.299	0.995	11.631	0.183
Ear diameter	5.912	2.542	19.662	2.814	0.061	1.772
Number of grain rows per ear	10.429	0.653	10.670	0.001	7.464	0.026
Spindle diameter	9.401	4.156	4.451	6.809	5.302	0.242
Seed length	4.049	0.722	26.502	0.004	0.506	0.042
Seed width	8.078	2.336	0.000	0.230	20.000	5.634
Seed thickness	0.935	0.498	9.313	11.115	18.741	23.873
Thousand-Kernel weight	4.251	2.660	8.735	9.325	17.132	1.140
Hectoliter weight	15.404	0.866	2.831	0.006	0.336	3.309
Eigenvalue	5.003	3.425	2.706	2.043	1.212	0.767
Variability (%)	29.430	20.149	15.920	12.016	7.127	4.513
Cumulative %	29.430	49.579	65.499	77.515	84.642	89.155

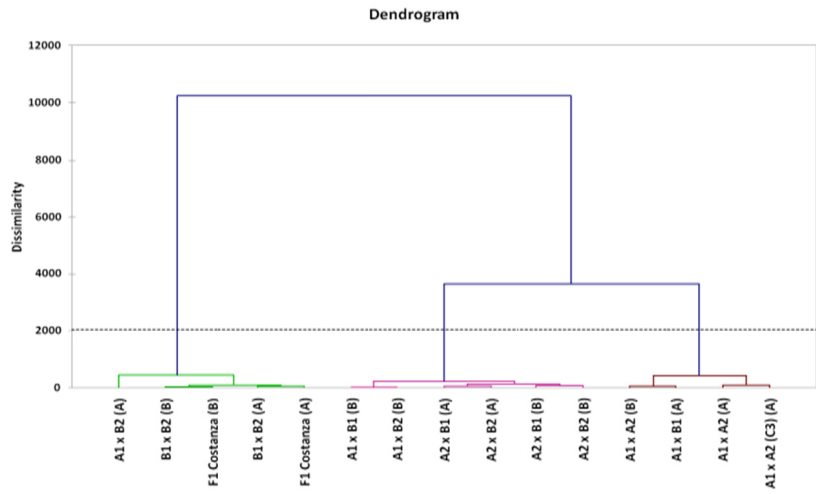

Figure 1. Dendrogram using agglomerative hierarchical clustering (AHC) for maize crosses based on 17 quantitative and quality traits

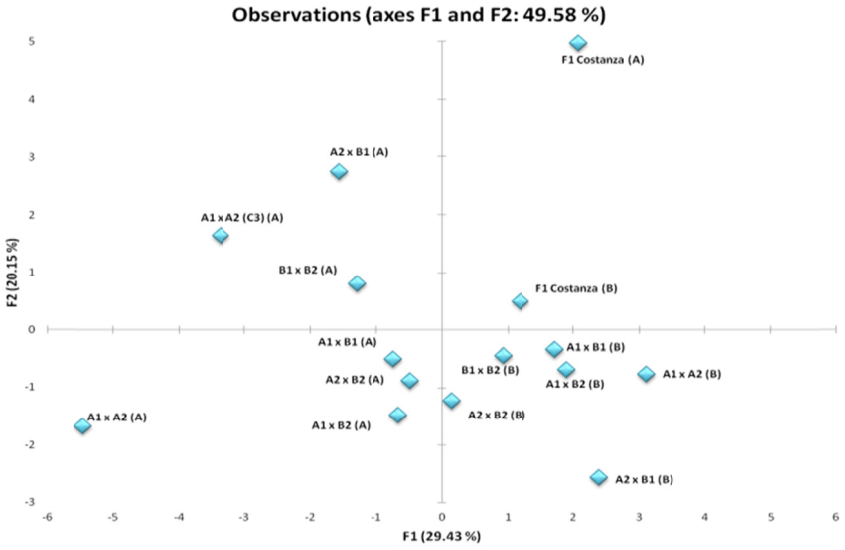

Figure 2. Principal Component Analysis of 17 quantitative and quality traits

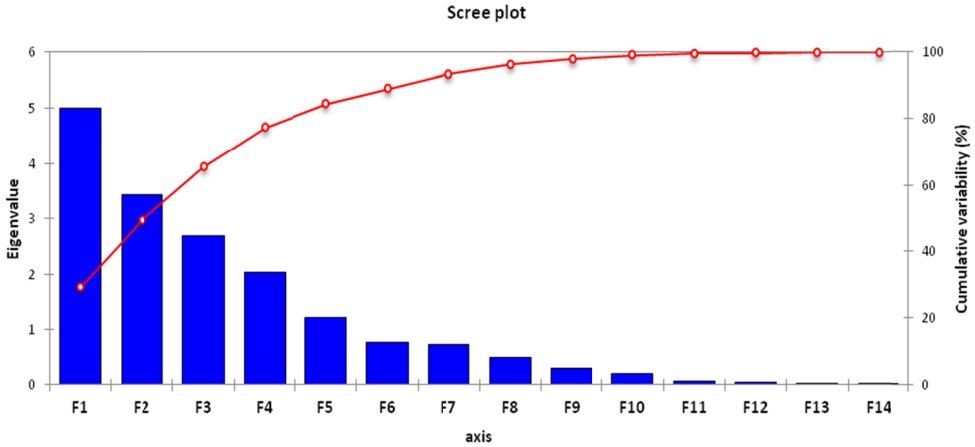

Figure 3. Screen plot of eigenvalues obtained from the PCA process for 17 quantitative and quality traits

3.2 Molecular Analysis With ISSR Markers

A total of 64 polymorphic bands were obtained from the 6 primers. 100% of all loci were polymorphic. Gene diversity ranged from 0.268 to 0.383 and Shannon Index (I) from 0.320 to 0.567 (Table 6). In other studies, polymorphism was found over 70% for maize populations (Carvalho, Ruas, Ruas, Ferreira, & Moreira, 2002; Žiarovska et al., 2013) or near 70% for maize inbred lines (Idris, Hamza, Yagoub, Ibrahim & El-Amin, 2012). It is possible that differences in such genetic materials may be due to polymorphism that acts as dominant genetic markers in case of populations (Sperisen & Bücher, 1998; Žiarovska et al., 2013).

The first two coordinates of the PCA (Figure 4) represented only the 34.49% of the total heterogeneity among the maize crosses, while the first coordinate explained 18.49% of the variation. The distances on the PCA are corresponsive to the genetic distances. The F1 Costanza checks were placed on the same spot in PCA plot. On the other hand, the six newly-developed crosses evaluated in different environments showed measurable distances between identical lines. These distances indicate genotype-environment interactions. Genetic differentiation was observed between the six newly-developed crosses grown in agronomically contrasting environments (A and B). Generally, genetic differentiation can be attributed to the effect of both abiotic and biotic environmental factors on distribution patterns and populations genetic structures, as measured by random dominant markers (Odat, Jetschke, & Hellwig, 2004, Abraham et al., 2015).

Table 6. Polymorphic bands, polymorphism percentage (%), shannon index (I), Gene Diversity (GD), based on six primers in ISSR analysis

Primer name	Polymorphic Bands	Polymorphism Percentage (%)	Shannon Index (I)	Gene Diversity (GD)
UBC834	9	100	0.417	0.277
UBC824	9	100	0.376	0.268
UBC841	7	100	0.415	0.282
UBC807	19	100	0.567	0.383
UBC811	12	100	0.522	0.365
UBC827	8	100	0.32	0.232
Mean	10.66	100	0.436	0.301

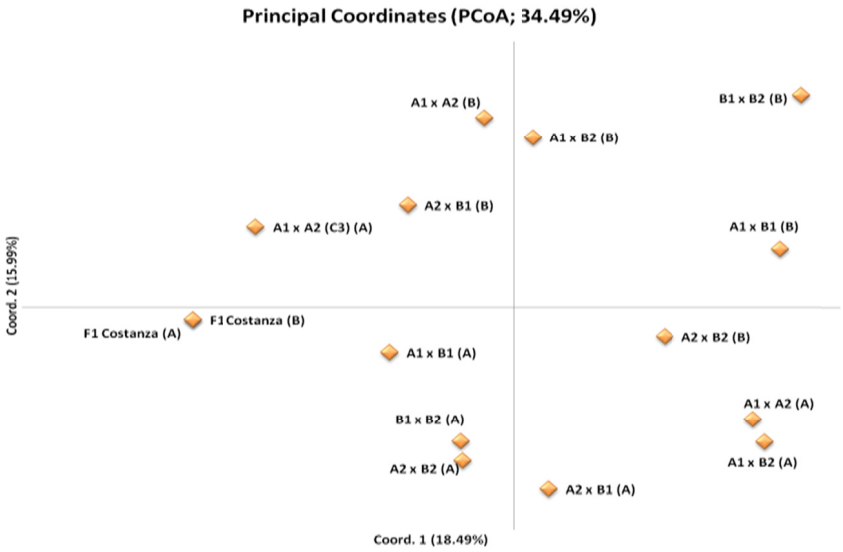

Figure 4. PCA analysis of genetic diversity of maize crosses

4. Conclusions

According to PCA analysis, the six newly-developed crosses, which were evaluated in different environments, showed measurable distances between the identical lines (B1×B2 (A) and B1×B2 (B)), suggesting

genotype-environment interaction. Also, cluster analysis showed that some crosses are grouped in separate and distinct clusters, indicating that dissimilar developmental environments may cause changes in quantitative traits. This may be due to the origin of the selected lines, since they were developed in different locations. Lines developed in the same location gave crosses that had similar behavior in the two location. Also, it is clear that a kind of gene fixation is apparent from the C3 cycle in A1×A2 cross, since it is close (and similar) to the C4 cross. Crosses including A2 line showed a greater stability in both environments.

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