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GENETIC DIVERSITIES IN THE SIXTH - GENERATION OF SELECTION (S_6) OF SOME INBRED LINES OF MAIZE BASED ON THE PHENOTYPIC CHARACTERS AND SSR

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ABSTRACT

The main objective of this research was to study the diversity of maize inbred lines based on phenotypic characters and SSR markers. The research, which was conducted from February to June 2011, was divided into field and laboratory research for molecular analysis. The molecular analysis was conducted at the Biotecnology Laboratory, Faculty of Agriculture, Brawijaya University Malang. The field trial was done in Kandat, Junrejo, Batu. Materials of the research were 35 genotypes of maize. Steps for molecular analysis included: (1) DNA isolation, (2) DNA-quality test, (3) PCR SSR, and (4) Visualization of the amplification result. The field trial was done by planting genotypes using a RCBD with 2 replications. Result of the dendogram analysis showed that 35 genotypes of maize were divided into 2 main clusters, A and B, that having 35% similarity level. Result for the analysis of variance showed significant difference among genotypes of maize on the entire of the observed phenotype characters. Heritability (H) ranged from 61.8% to 98.1%. Coefficient of variance ranged from 0.71 to 7.20%. Analysis on the genotypic correlation ranged from 0.06 to 1.0. Numbers of rows per ear showed significant correlation, in which Jaccard's similarity coefficient (r_s) = +0.43.

Keywords: genetic diversity, phenotype, molecular, inbred line, maize.

INTRODUCTION

It is important to recognize the genetic diversity of inbred lines particularly in maize breeding program. Higher genetic diversity in basic population is an important material in creating superior variety (Srdic *et al.,* 2007; Sofi *et al.,* 2011). Basic population that has higher genetic diversity provides greater opportunities to obtain more cross-combinations and better response to selection.

The genetic diversity of maize inbred lines was tested by observing the phenotypic characters. Along with the development of plant molecular biology, the genetic diversity was tested using molecular marker on DNA level. The important DNA-markers include RFLP (*Restriction Fragment Length Polymorphism*), RAPD (*Randomly Amplified Polymorphic DNA*), AFLP (*Amplified Fragment Length Polymorphism*), STS (*Sequence Tagged Sites*), SCARs (*Sequence Characterized Amplified Regions*), SSRs (*Simple Sequence Repeats*) or microsatellites, and SNPs (*Single Nucleotide Polymorphism*) (Ye-yun *et al*., 2005; Azrai, 2006). Those markers are used in accordance with the need of plant breeding (Azrai, 2006; Ye-yun *et al.,* 2005). SSR marker is used to identify and verify a plant variety. Yamasaki *et al.* (2005) used SSR marker to study the genetic diversity in maize inbreds, landraces and teosintes, and as a result, they found significant genetic difference among those 3 populations. Yashitola *et al.* (2002) detected the purity of paddy hybrid using microsatellite marker and STS (*Sequence Tagged Sites*) and the result showed that microsatellite was better than STS in determining the germplasm purity in paddy hybrid.

Microsatellite or Simple Sequence Repeats (SSRs) is mostly used for genetic mapping and genetic diversities analysis on inbred lines (Yamasaki *et al*., 2005; Pabendon *et al*., 2007; Trindade *et al.,* 2010). Microsatellite can be utilized to characterize inbred lines on DNA level, but

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accurate conclusion cannot be drawn without any phenotypic data supports. Therefore, some comprehensive research on molecular and phenotypic testing is required to use the SSR marker efficiently, particularly in exploiting the genetic diversities of germplasm and setting the heterotic groups.

Besides studying the genetic diversities in a population, the molecular marker can be used as Marker Assisted Selection (MAS), such as: to identify the parent lines accurately in order to improve specific character, to find the trace of appropriate alleles in each generation, and to identify the individual according to both favored quantitative and qualitative characters (Azrai, 2005; Lee *et al*., 2008). The molecular marker can be used for Marker Assisted Selection and to choose the targeted genes in selection correctly. The use of molecular marker will be effective for Marker Assisted Selection when strong relationship exists between the molecular data and the specific phenotypic characters.

MATERIALS AND METHODS

Genetic Material

The research used 35 genotypes of maize materials that consisted of 33 inbred lines of the sixth-generation of selection (S_6), such as: G-01, G-02, G-03, G-04, G-05, G-06, G-08, G-11, G-13, G-14, G-15, G-17, G-18, G-19, G-20, G-21, G-23, G-24, G-26, G-34, G-35, G-36, G-38, G-40, G-42, G-44, G-46, G-49, G-51, G-BO,G-T00, G-T14, G-T15, G-T22, G-T37, which derived from selfing and sibmating of both commercial and local varieties in Indonesia. As comparison, 2 open pollinated varieties (OPV) were applied: Bisma and Lamuru.

SSR Analysis

Samples of the research were 10 plants from each genotype. Samples were taken from young leaves, 25-35 days after planting. DNA isolation was applied using CTAB method (Doyle and Doyle, 1987; Kostava *et al*., 2006) which was modified using an active carbon (Krizman, 2006). Measurement of the DNA quality used was electrophoresis, in which 1% agarose was dissolved in TBE 0.5x. 0.4 g of agarose was dissolved in 40 ml TBE and then poured into the plate and allowed it to harden. After that, the gel submerged in the electrophoresis chamber. The sample used 2 µl DNA and 1 µl loading dye, which were put into wells. The electrophoresis was performed under 100 volt for about half an hour. The result of such electrophoresis was visualized over the ultraviolet light.

PCR reaction optimization program was performed before amplification. Optimization was performed to obtain PCR optimum condition for DNA amplification using primer microsatellite, which had been previously determined. Also, it was performed to choose primer, which had higher polymorphism. PCR reaction optimization program was: 1 initial denaturation cycle under 94 ^{o}C for 4 minutes, followed by 37 denaturation cycles under 94 ^{o}C for 50 seconds and annealing temperature of 55 ^{o}C for 1 minute. PCR cycle was completed by 1 final extension cycle under temperature of 72 ^{o}C for 1 minute. This optimization used 9 pairs (Table 1).
Electrophoresis was applied to study the DNA amplification using PCR. One percent of agarose was dissolved in 40 ml buffer TBE 0.5x, then it was electrophoresized under 100 V for 30 hours. Then, the gel was submerged in EtBr (Ethidium Bromide) for 15 minutes. The visualization was performed over ultraviolet.

Field Trial

The experiment used 35 genotypes of maize: 33 inbred lines and 2 comparative varieties which were planted using Randomized Complete Block Design with 2 replications. The plants were grown on 5 m plots, and the planting distance was 0.75 m between rows and 20 cm within a row. Sample for each replication comprised 20 plants. Dosages of fertilizers were 300 kg NPK/Ha and 100 kg Urea. The fertilizers were given 4 times in different period as follow : (1) the first fertilizer was given during the initial planting (0 day after planting), for about 100 kg NPK/Ha, (2) the second fertilizer was given within 21 days after planting, for about 75 kg NPK/Ha and 50 kg Urea/Ha, (3) the third fertilizer was given within 45 days after planting, for about 75 kg NPK/Ha and 25 kg Urea/Ha, (4) the fourth fertilizer was given within 60 days after planting, for about 50 kg NPK/Ha and 25 kg Urea/Ha.

Table 1. Primer names used in study (Wu *et al.,* 2006)

Primer name	Forward sequence (5'-3')	Reserve sequence (5'-3')
P-umc1165	TATCTTCAGACCCAAACATCGTCC	GTCGATTTTCCCGATGTTAAA
Nc030	CCCCTTGTCTTTCTTCCTCC	CGATTAGATTGGGGTGCG
Umc1294	GCCGTCAACGGGCTTAAACT	GCCTCCAGCTCTCTCGTCTCT T
ZTC161	GCTTTCGTCATACACACACATTCA	ATGGAGCATGAGCTTGCATATTT
Phi001	TGACGGACGTGGATCGCTTCAC	AGCAGGCAGGAGGTCAGCAGCG
Phi034	TAGCGACAGGATGGCCTCTTCT	GGGGAGCACGCCTTCGTTCT
Phi057	CTCATCAGTGCCGTCGTCCAT	CAGTCGCAAGAAACCGTTGCC
Phi080	CACCCGATGCAACTTGCGTAGA	TCGTCACGTTCCACGACATCAC
Phi119	GGGCTCCAGTTTTCAGTCATTGG	ATCTTTCGTGCGGAGGAATGGTCA

33 inbred lines and 2 comparative varieties were grown in polybags. The medium was fine sands mixed with manures. Polybags were used to eliminate the plant loss and avoid any disease and pest attack during the initial growth since the planting was conducted during rainy season. The plantlets transferred to the field within 10 days after planting. Nine agronomic characters of the maize were observed, such as: the plant height (cm), leaf length (cm), days of pollen emergence (day), harvest time (day), kernel weight per plant (kg), diameter of the ear (cm), number of rows per ear, and kernel water content in harvest time (%), as well as the grain yield (kg per ha).

Data Analysis

Analysis of Variance Based on Molecular Data

Data from photo-documentation was DNA band pattern as a result of PCR amplification using primers SSR. DNA band resulting from amplification was interpreted as qualitative data by observing the presence and absence of such DNA band, which was transferred to binary data through scoring on one position of the equivalent band row. Scoring is taken according to the band, for instance: if there was a band, the score was one (1) and if there was no band, the score was zero (0). This data was used to calculate genetic similarity coefficient and to construct dendogram.

Genetic relationship was determined by Jaccard's similarity coefficient with the formula: Sj = A/(A+B+C), where Sj: Jaccard's similarity coefficient, A: numbers of the DNA band (allele) which belongs to both genotypes 1 and 2, B: numbers of specific band belonging to genotype 1, and C: number of specific band belonging to genotype 2 (Sneath and Sokal (1973) in Cordeiro *et al.,* 2003). Relationship of the inbred lines correlations was determined by genetic similarity analysis. Those inbred lines were categorized according to the genetic similarity matrix through UPGMA (Unweighted Pair Group Method Using Arithmetic Average) using NTSYSpc (Numerical Taxonomic System) program version 2.02i.

Analysis of Variance Based on Phenotype Characters

Data of the trial results on the inbred lines diversity testing was analyzed using analysis of variance, and the linear model was as follows: $X_{ijkl} = \mu + g_{ijk} + b_l + \sum_{ijkl}$, whereas: X_{ijkl}= value of the observed characters on the genotype-*ijk* and the group-*l*, μ = influence of the population median on the observed characters, g_{ijk}= influence of the genotype-*ijk* on the observed characters, b_l = influence of the group-*l* on the observed characters, $\sum_{ijkl}$ = influence of the trial error on the genotype-*ijk* and group-*i.* In order to find out the percentage of the genetic role which influenced the phenotype performance, a heritability prediction was applied using variance component by the formula: $H = (\sigma^2_g/\sigma^2_f)$ whereas: σ^2_g = genetic variance and σ^2_f = phenotype variance. Close relationship among the observed characters was analyzed using simple correlation analysis from Singh and Chaundary (1977). Formula for the genotypic correlation coefficient between two phenotypic characters was as follows: $rg(x_1\ x_2)$ = Cov

$(x_1.x_2)/\sqrt{\square\square^2(x_1).\square^2(x_2)}$, where rg($x_1$ x_2) = relation of genetic coefficient between characters of x_1 and x_2, Cov ($x_1.x_2$) = covariance between the characters of x_1 and x_2, $\square^2(x_1)$ = variance of x_1 and $\square^2(x_2)$ = variance of x_2.

Correlation Analysis between Molecular Data and Phenotype Data

Relationship between molecular and phenotype data was analyzed using Spearman's rank correlation coefficient 1904 (Singh and Chaundhary, 1979; geographyfieldwork, 2011; udel. edu.2011) by the formula as follows: $r_s = 1-[(6d_i^2)/n(n^2-1)]$, where r_s = Spearman's Rank correlation coefficient value, d = difference between ranking on the first and the second data, and n = numbers of treatment.

RESULTS AND DISCUSSION

Genetic Variability Based on The Molecular Marker

Three out of 9 primers used had higher polymorphism, such as: umc1294, ZCT161 and phi080. Senior *et al.* (1998) applied the microsatellite marker in maize and it showed higher polymorphism. Those three primers were used to estimate the genetic distance of 33 inbred lines and 2 open pollinated varieties (OPV). The result of dendogram analysis showed that 35 genotypes of maize were divided into 2 main clusters, A and B, in which the similarity level was 35%. Cluster A comprised 33 genotypes and cluster B comprised 2 genotypes. Cluster A was divided into 2 sub-clusters such as: A_1 and A_2. Sub-cluster A_1 comprised 11 genotypes, while sub-cluster A_2 comprised 22 genotypes (Figure 1).

In cluster A and B, genetic similarity of 35% was obtained, in sub-clusters B_1 and B_2, the genetic similarity was about 42%. Sub-cluster A_1 comprised 6 groups: Group I (G-01), II (G-04 and G-49), III (G-23), IV (G-36, G-15, G-42, G-B0, G-46), V (Bisma), and VI (G-T00). Sub-cluster A_2 comprised 7 groups: VII(G-02, G-18, G-19, Lamuru), VIII (G-07, G-13, G-17, G-40, G-T22), IX (G-44), X (G-08, G-34, G-38, G-51), XI (G-11, G-20, G-T14, G-24, G-26, G-35), XII (G-06), and XIII (-T37). Cluster B comprised 2 groups in which each genotype just had one genotype: Group XIV (G-03) and XV (G-21) (Figure 1).

Lamuru was an open pollinated variety and belonged to group (VII), along with G-02, G-18 and G-19 that had genetic similarity level of 100%. It was estimated that G-02, G-18 and G-19 were inbred lines which were developed from Lamuru variety so that they have higher genetic similarity. Bisma and G-T00 were included in one sub-cluster B_1, in which the genetic similarity was 75%. Both genotypes, which were suggested having close relationship due to G-T00, were developed from commercial variety in Indonesia, which possibly had identical genealogy with Bisma. Mohammadi *et al.* (2008) reported that the success in using molecular marker to observe the genotype variability and estimate the genetic distance was determined by numbers of primer, hereditary pattern, uniformity distribution in genome and specific information on each used primer. Dhliwayo *et al*. (2009) stated that categorization was based on molecular marker which can be used as utilization directory of germplasm in smaller group or as initial grouping of germplasm from uncharacterized accessions.

Genetic Variability Based on the Phenotype Characters.

Result of the variance analysis showed significant difference among genotypes of maize in the entire observed phenotype characters. Heritability (H) showed higher value in all observed phenotype characters. Variability coefficient ranged from 0.71 – 7.20% and showed that the experiment was accepted (Table 2).

Analysis of variance on genotype showed significant difference on all observed phenotype characters. It showed greater variability in characters of the plant height, leaf length, days of pollen emergence, harvest time, kernel weight per plant, diameter of the ear, number of rows per ear, kernel water content in harvest time, and the grain yield. Without such higher genetic diversity, it would be difficult to improve the plant characters in breeding program (Fehr, 1994). The grain yield showed variability among the tested genotypes, G-18 had the highest grain yield and G-46 had the lowest grain yield. The genotype variability was based on the grain yield character (Figure 2).

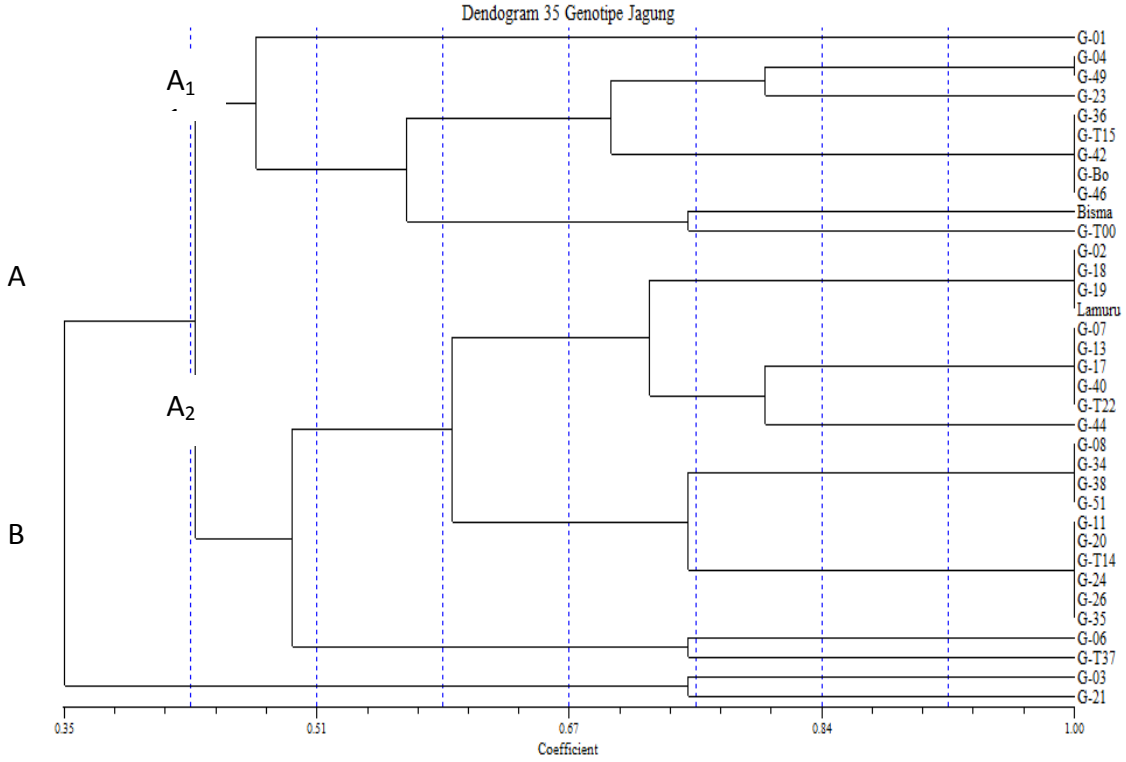

Figure 1. Dendogram of 35 genotypes of maize based on SSR marker using UPGMA method and constructed based on Jaccard's similarity coefficient

Table 2. Analysis of variance on phenotype characters of maize, Coefficient of Variance (CV) and Heritability (H)

Characters in maize Degree of freedom	**Replication**	**Genotype**	**Pooled error**	**CV (%)**	**H**
	1	**34**	**34**		
Plant height	274.6	813.6*	78.82	4.90	82.3
Leaf length	0.2	154.1*	13.17	4.00	84.3
Days of pollen emergence	0.3	33.8*	0.32	0.98	98.1
Days of harvest time	1.2	36.5*	0.51	0.71	97.2
Kernel weight plant^{-1}	0.0001	0.0025*	0.00006	7.20	95.3
Diameter of ear	10.3	58.1*	6.04	6.40	81.2
Number of rows ear^{-1}	0.2	3.4*	0.31	4.30	83.3
Kernel Water content in harvest time	5.6	10.0*	2.36	6.90	61.8
Grain yield	99094.9	9267548.6*	169242.62	6.30	96.4

Remarks: * Significant at P<0.05, respectively

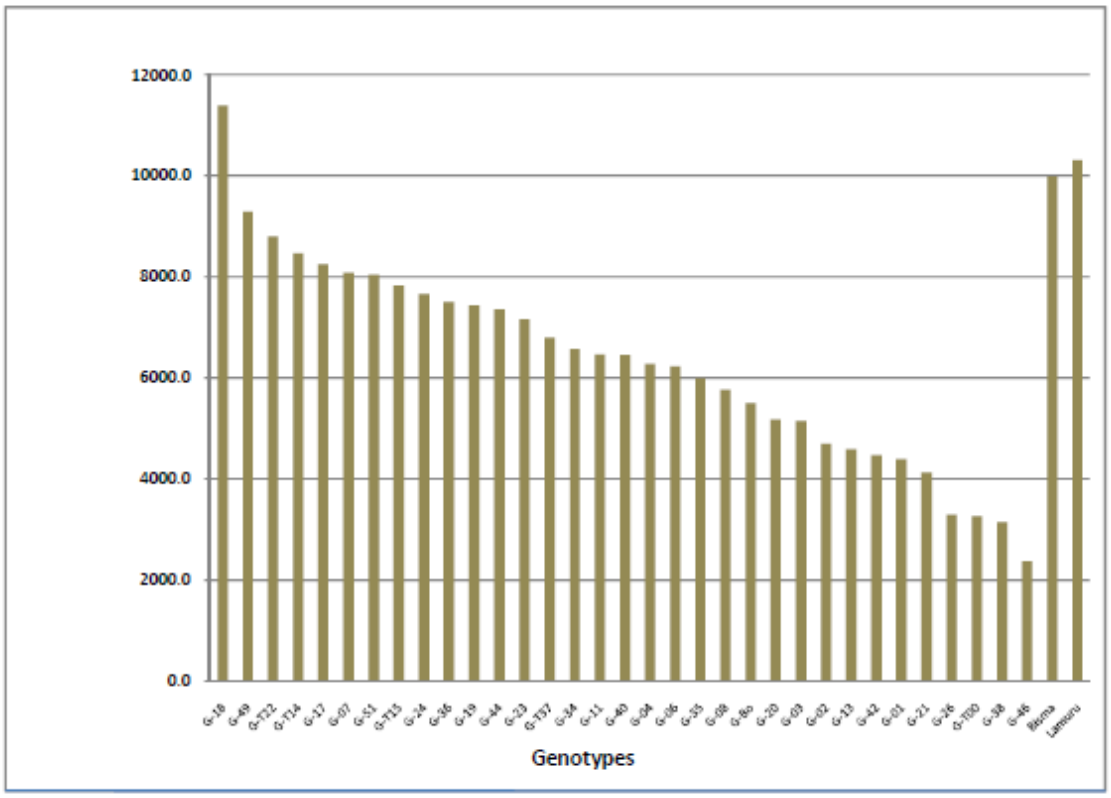

Figure 2. Grain yield in 35 genotypes of maize

Genotypic Correlation among the Observed Phenotypic Characters

Genotypic correlation analysis showed significant and strong relationship from one phenotype character to another phenotype character of the plant (Table 3).

The result of the research showed that the grain yield had genotypic correlation with the leaf length, days of pollen emergence, harvest time, kernel weight plant^{-1}, diameter of the ear, number of rows ear^{-1}, kernel water content in harvest time, and the grain yield in harvest time. The highest genotypic correlation was in the kernel weight plant^{-1} and the grain yield. Number of rows ear^{-1} showed significant correlation with leaf length, days of pollen emergence, harvest time, kernel weight plant^{-1}, diameter of the ear, and the grain yield. Salami *et al.* (2007) stated that significant correlation was apparent on several phenotype characters of some tested genotypes of maize.

Correlation between Molecular Data and Phenotype Data

Relationship between molecular data and the phenotype characters of the plant used Spearman's rank correlation. Spearman's rank (r_s) correlation coefficient value between the phenotype characters and Jaccard's similarity coefficient is presented in Table 4. Number of rows per ear^{-1} showed significant correlation with Jaccard's similarity coefficient (r_s) = +0.43. Therefore, it was suggested that some relationship between primers applied as molecular marker and the phenotype traits on 35 tested genotypes of maize. The significance graphic is presented in Figure 3.

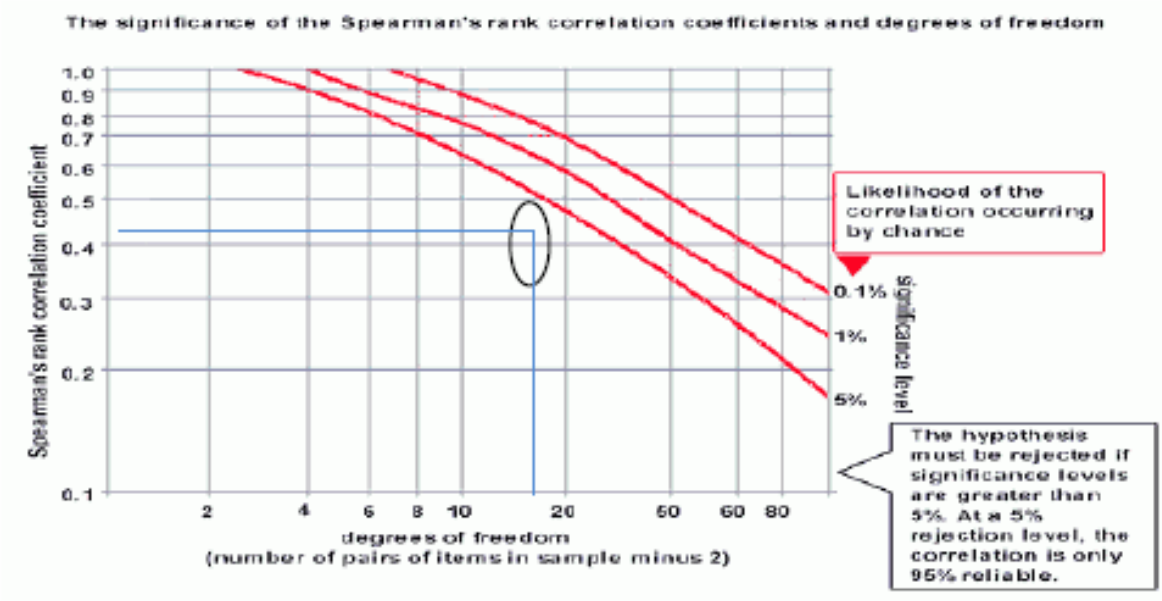

Figure 3. Significance graphic on Spearman's rank correlation coefficient

Table 3. Genotypic correlation between the grain yield and other Characters

The Plant Characters	1	2	3	4	5	6	7	8	9
Plant height (1)	1	0.63*	0.17	0.12	0.35	0.4	0.3	0.06	0.36
Leaf length (2)		1	0.62*	0.63*	0.65*	0.41	0.63*	0.58*	0.63*
Days pollen to shed (3)			1	0.80*	0.53*	0.37	0.72*	0.62*	0.5*
Days of harvest time (4)				1	0.69*	0.55*	0.63*	0.46*	0.67*
Kernel weight plant^{-1} (5)					1	0.49	0.77*	0.31	1.00*
Diameter of ear (6)						1	0.78*	0.12	0.77*
Number of rows ear^{-1} (7)							1	0.52*	0.75*
Kernel Water content (8)								1	0.27
Grain yield (9)									1

Other facts showed that genotype correlation among the plant characters described significant genetic correlation between number of rows ear^{-1} and other phenotype characters. Therefore, it was presumed that there was some relationship between primers used as molecular marker and other phenotype characters. The phenotype characters, particularly, had genetic correlation with number of rows ear^{-1}, such as: leaf length, days pollen to shed, days of harvest time, kernel weight plant^{-1}, diameter of ear, and grain yield. Aguiar *et al*. (2008); Pabendon *et al.* (2009) categorized the inbred lines using molecular marker as a way to classify the genotypes instead of the conventional one. The molecular marker can be applied to predict the genetic diversity of the plant (Aquilar *et al*., 2008; Pongsai *et al*., 2009: Trindade *et al*. 2010) and it can be used to select the molecular assistance in maize (*Marker Assisted Selection)* (Azrai, 2005; Lee *et al*., 2009).

Table 4. Spearman's rank correlation coefficient value between the Phenotype Characters and Jaccard's similarity coefficient

Phenotype Characters of Plant	r_s
Plant height	0.28
Leaf length	0.24
Days pollen to shed	0.02
Days of harvest time	0.04
Kernel weight per plant	0.24
Diameter of ear	0.08
Number of rows per ear	**0.43***
Kernel water content in harvest time	0.21
Grain yield	0.36

CONCLUSIONS AND SUGGESTION

Based on result of the research, some conclusions were drawn as follows: (1) the genetic diversities were found based on the phenotype characters and the molecular marker, (2) the genetic correlation was found among the observed phenotypic characters, and (3) strong and significant correlation between Jaccard's similarity coefficient and number of rows ear character was also found. Therefore, it was presumed that there was some relationship between primers used as molecular marker and other phenotypic characters, particularly the phenotype characters, which had genetic correlation with number of rows ear^{-1}, such as: the leaf length, days of pollen emerging, harvest time, kernel weight plant^{-1}, diameter of ear, and the grain yield. Therefore, number of rows ear^{-1} can be used as one of the selection criteria in breeding maize since it showed strong and significant correlation between phenotypic characters and molecular data as well as higher heritability value. For further review, it is suggested to learn more about relationship among 3 primers and the previous generation and after S_6 as well as multiply number of primer used in learning the genotypic diversity in maize.

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