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Using single morphological and RAPD molecular markers to classify a segregating generation of wheat (*Triticum aestivum* L.) into two earliness groups

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Abstract

An F_2 population derived from crosses between variety Faisalabad and line FM36 used in the present study at Sharekord University, Iran in order to investigate the ability of RAPD and qualitative markers to classify the F_2 individuals for earliness. Random Amplification of Polymorphic DNA analysis (RAPD) revealed that among 30 primers tested, 8 primers indicated polymorphisms in F_2 individuals and only two primers (OPM35 and OPG12) indicated polymorphisms in both parents and the F_2 population. The F_2 individuals were classified into two groups based on the presence or absence of the polymorphic bands and also based on two qualitative traits (hairy glumes and growth habit at the early stages), separately. The F_2 individual indicated large variations for days to heading. The results obtained using t-test revealed that sub-populations classified by the presence or absence of the polymorphic band amplified using OPM35 were significantly different for heading date implying the ability of this band for being used as an indirect selection of heading date. On the other hand, the means of the sub-populations classified by presence or absence of the band amplified using OPG12 were significantly different for days to maturity. Classifying the F_2 individuals based on the form of growth at the early stages resulted to two sub-populations, one having erect and the other rosette form of growth. These two sub-populations were significantly different for grain weight, days to heading and days to maturity. Conversely, when the F_2 individuals classified into two sub-populations based on having or lacking hairy glumes, they did not indicate any difference for the traits under study.

Keywords: earliness, molecular marker, morphological marker, segregating generation, wheat.
Abbreviations: DH: days to heading; DM: days to maturity; GY: grain yield; HI: harvest index.

Introduction

Quantitative complex traits in wheat possesses lower heritability compared to qualitative traits or/and molecular markers (Poehlman, 1995). Broadening the genetic base of common wheat by identification and introgression of potentially useful genes from progenitor species is an important germplasm development goal (Ladizinsky, 1985). Phonological characters such the time of spike appearance, days to heading (DH), days to maturity (DM) and even vernalisation requirement indicate quantitative inheritance and low heritability (Kato et al., 2000; Snape, 1987). Differences between developmental stages among wheat genotypes exist worldwide. The characters related to developmental stages affect wheat production and adaptation (Slafer et al., 1999) and major differences in DH among wheat genotypes can contribute to variation for GY and even to variation for physiological characters during the life cycle. Since the screening for natural earliness requires to be conducted under full satisfaction of the photoperiod requirements, they are mostly conducted under controlled conditions such as green houses and growth chambers. Under these controlled conditions, in which for practical and economic reasons, temperatures are substantially higher than those actually experienced in the filed by the crop (Appendino and Slafer, 2003). As the result, screening the genotypes for earliness is difficult due to complexity of the trait and effects of environmental factors such as high temperatures. It is now proved that the complex traits such as GY, HI and earliness are under quantitative control and their effects on genetic variation are relatively small (Yano and Sasaki, 1997). Earliness, however, is less environmentally sensitive and has higher heritabilities than GY (Bezant et al., 1997; Yano and Sasaki 1997). While looking for genetic factors controlling grain yield, genes for yield components should also be determined to provide more information that is useful. One of the traits that affect yield components is vernalisation requirement. Vernalisation genes regulate the requirements of exposure to cold temperatures and it is reported that vernalisation insensitivity promote heading and flowering time (Bullrich et al., 2002). In recent years, considerable emphasis has been placed on the development of molecular or morphological markers for different objectives. Identification of molecular bands or morphological markers associated with earliness or grain weight is the most important step in selecting genotypes having higher yield at the early stages of growth. This is because of the fact that identification of high yielding or early genotypes at the end of growth stage is costly and time-consuming and so scientists have been trying to establish relationships between markers on the molecular or phenotypic levels and quantitative traits such as grain yield. This relationship will help the plant breeders to select for quantitative traits in segregating generation where assessing the genotype is difficult (Diers et al., 1996). Some investigations in maize and oilseed rape have shown that the genetic diversity of parents for restriction fragment length polymorphism (RFLP) markers was significantly correlated

Table 1. Elements of polymerase chain reaction

Elements of reaction	Concentration	Quantity	Final concentration
PCR Buffer	10×	2.5µl	1×
dNTPs	10mM	0.5µl	200µM
$MgCl_2$	50mM	0.75µl	1.5mM
Primer	5 ng/ µl	3µl	15ng
Taq DNA Polymerase	5u/ µl	0.12µl	0.6u
dd H_2O	-	16.13µl	-
DNA	25ng/ µl	2µl	50ng

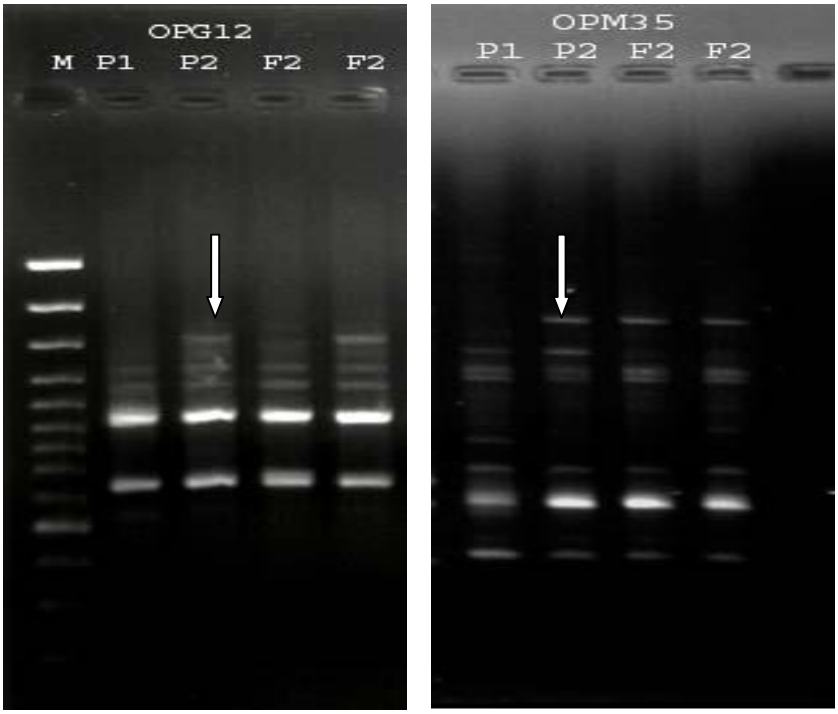

Fig 1. Bands showing polymorphism between parents and F_2 individuals (only two plants of F_2 population are shown) (the arrows indicate polymorphic bands)

with hybrid performance and that yield could be predicted by using molecular markers (Smith et al., 1990). In contrast, some other studies showed that the correlation of hybrid performance with molecular diversity between parents was too low to be of any predictive value (Lee et al., 1989; Godshalk et al., 1990; Dudley et al., 1991). Nonetheless, the automation of (PCR) offers a means to mitigate the time, expense, and safety hazards associated with RFLPs. A single, short DNA primer sequence can direct the amplification of a specific DNA sequence via PCR. These random amplified polymorphic DNA sequences (RAPDs) have shown some promise as molecular markers in wheat (Fritz et al., 1995) so that RAPD markers (He et al., 1992) can effectively differentiate genotypes in wheat. Wheat cultivars with a winter habit, which do not have any gene for vernalisation insensitivity, are called winter wheats and they require considerable vernalisation for initiating the reproductive stage. On the other hand, wheat cultivars with a spring habit, which do not respond to vernalisation, are called spring wheats. There is another group of wheat cultivars, which are only partly sensitive to vernalisation. These cultivars respond to vernalisation and start the reproductive stage earlier when they receive enough cold treatment during the growth cycle. These cultivars are called semi-winter. The latter type of genotypes, remain vegetative in a rosette form when they are planted in spring without vernalisation. Rosette growth caused due to shortage of vernalization requirement can be easily identified in segregating generations and thus suitable markers for indirect selection if they appear to be linked with quantitative traits. Hairy glumes is also a widely distributed characters in wheat genotypes and suitable for being used as a morphological marker (McIntosh et al., 1998). Although genes controlling qualitative traits such as disease and insect resistances are generally targeted in germplasm development programs (Dweikat et al., 2004), genes for quantitative traits may be affected by environmental conditions and hence difficult to screen genotypes for quantitative traits themselves. The most important role of any genetical marker (including molecular or morphological markers) is to help breeders for indirect selection of quantitative traits.

Table 2. List of polymorphic primers and number of amplified bands.

Primer name	Number of Amplified bands	Number of Polymorphic bands	Percentage of polymorphic bands
OPG10	5	1	20.0
OPG12	4	1	25.0*
OPG15	8	1	12.5
OPG38	7	1	14.3
OPG43	6	1	16.6
OPM10	5	2	40
OPM35	5	1	20*
OPM23	6	1	16.6
Sum	46	9	19.5
Average	5.75	1.125	20.6

*: These primers indicated polymorphism in both the parents and the F_2 population

Table 3. The means and differences between the two parents of the F_2 Population for the characters under study

Trait	Faisalabad	FM36	Difference
Days to heading	71.63	89.23	17.60**
Spike length (cm)	9.51	7.24	2.27**
Grain yield/plant (gr)	4.12	1.82	2.32**
Harvest index	0.23	0.14	0.09**
Days to maturity	105.61	115.26	9.65**

*and **: significant at 5% and 1% level of probability, respectively.

Table 4. The differences observed between the sub-populations classified based on the presence or absence of the polymorphic band amplified using OPM35 for the quantitative traits under study

Trait	Presence	Absence	Difference	P value
Days to heading	79.62	86.77	-7.15	0.020
Spike length (cm)	8.18	7.62	0.56	0.310
Grain yield/plant (g)	3.22	2.16	1.06	0.200
Harvest index	0.18	0.16	0.02	0.460
Days to maturity	111.48	113.42	-1.96	0.220

The breeders can meet the aim if the molecular markers are able to differentiate between the populations having the markers and those lacking them. This study was designed to investigate the characteristics of agronomic quantitative values in different groups clustered based on the presence or absence of RAPD and morphological markers in an attempt to see how RAPD markers or morphological markers could be reliable as indirect selection criteria for screening quantitative traits.

Results

Classifying the F_2 population based on molecular and morphological markers

Of the 30 primers tested, 22 amplified monomorphic bands, 6 primers showed polymorphism in F_2 population only, and two bands showed polymorphism both in the parents and in the F_2 population (Table 2). Primers OPG12 and OPM35 amplified two polymorphic clear bands (each one) differentiating between the two parents and F_2 individuals. As can be seen from the Figure, Fisalabad (signed as P_1 in Fig 1) had no polymorphic band but line FM36 (marked as P_2 in Fig 1) showed two polymorphism bands. The individuals of F_2 population were classified based on the presence of the polymorphic band amplified by OPG12 and OPM35 and based on hairy glumes and rosette shape separately. Classifying the F_2 individuals into two sub-populations based on the presence or absence of the polymorphic band amplified by OPM35 lead to 82 percent of individuals (144 individuals) having and 18 percent (36) lacking the band, respectively. In addition, from 180 F_2 plants, 110 showed the polymorphic bands amplified by OPG12 and the rest (70 plants) did not show this band. Recording the F_2 individuals for hairy glumes indicated that 136 out of 180 (76 percent) F_2 individuals had hairy glumes and 24 F_2 plants (24 percent) had hairless ones. From 180 F_2 recorded plants, 135 had erect and 45 had rosette shapes, respectively. In general, it was possible to classify the individuals of the F_2 population into two sub-populations using all the 4 markers**.**

Comparisons between sub-populations for quantitative traits

Primary data showed that, among 13 quantitative traits, the parents of the cross were significantly different for 5 characters only. The amounts of these 5 characters for the parents are presented in Table 3. The F_2 individuals were then assessed for the above 5 characters in an attempt to promote the chance of finding distinguished groups in the F_2 generation. The results comparing the means of the two sub-populations formed by having or lacking the band amplified by OPM35 are presented in Table 4. As can be seen from the table, DH for subpopulation having the band and that lacking the band were 79.66 and 86.77, respectively. The difference between the two means was significant for days to heading only. This implies that presence of the band is possibly linked to early appearance of spike and that presence or absence of the band has not been able to differentiate between the sub-populations for other quantitative traits. A more or less similar trend was also observed for sub-populations formed by the presence or absence of molecular band amplified using OPG12. The means of the sub-populations were not significantly different for all of the quantitative traits except

Table 5. The differences observed between the sub-populations classified based on presence or absence of OPG12 for the quantitative traits under study

Trait	Presence	Absence	Difference	P value
Days to heading	79.02	83.76	-4.74	0.060
Spike length (cm)	8.23	7.87	0.36	0.370
Grain yield/plant (gr)	3.54	2.55	0.99	0.150
Harvest index	0.19	0.17	0.02	0.380
Days to maturity	110.78	116.6	-5.82	0.010

Table 6. The differences observed between the sub-populations classified based on presence or absence of hairy glumes for the quantitative traits under study

Trait	Presence	Absence	Difference	P value
Days to heading	80.70	81.25	-0.55	0.770
Spike length (cm)	8.03	8.41	-0.38	0.780
Grain yield/plant (gr)	3.13	3.45	-0.32	0.680
Harvest index	0.19	0.16	0.03	0.400
Days to maturity	112.00	111.07	0.93	0.680

Table 7. The differences observed between the sub-populations classified based on growth habit for the quantitative traits under study

Trait	Erect growth	Rosette growth	Difference	P value
Days to heading	75.25	91.55	-15.63	0.000
Spike length (cm)	8.22	7.86	0.36	0.380
Grain weight /plant (gr)	4.11	1.46	2.65	0.001
Harvest index	0.23	0.11	0.12	0.001
Days to maturity	110.00	116.00	-6.00	0.000

for days to maturity (Table 5). The sub-population having the polymorphism band amplified by OPG12 and that lacking this band needed 110.78 and 116.60 days from planting to maturity, respectively. This result indicates a possible link between early ripening and the molecular band amplified by OPG12. A clear difference was also observed for the frequency of the two mentioned bands in the F_2 population. The above results indicated that the two primers are not able to differentiate between sub-populations for grain weight. The results of comparing means of sub-populations, formed based on the presence or absence of hairy glumes, are presented in Table 6. As can be seen from the Table, no significant difference was found between the sub-populations for quantitative characters under study indicating hairy glumes is associated with none of the characters. Despite the previous markers, sub-populations formed based on having or lacking rosette shape at the early stage of growth were different for GY, DM, DH and HI but not significant for spike length (Table 7). This implies that rosette genotypes reach heading stage and maturity stage later that erect ones. It is reported that rosette shape at the early stage of growth is due to vernalisation requirement in wheat when it is cultivating at the spring where cold treatments do not take place (Kainth, 1994). Therefore, cultivating the vernalisation sensitive genotypes in spring when temperature is not enough low to meet the vernalisation requirement, grain weight are also affected negatively. These results are similar with literature in some cases and in contrast with some other cases. Near isogenic lines (differing for vernalisation requirements only) were grown at spring in seven different areas by Stelmakh (1998) without artificial vernalisation. He reported that isogenic lines insensitive to vernalisation reached heading stage earlier and produced more grain weight and shorter spike length in comparison with sensitive ones. Thus, his results are similar to the present results in the case of effect of growth habit on grain weight and heading date and in contrast in the case of spike length. These inconsistent results may be due to environmental effects or interaction between temperatures and vernalisation requirement during growth stages (Roberts and Larson, 1985).

Correlation among the characters

Correlation coefficients between rosette shape and grain weight, days to heading and days to maturity were all significant (Table 8) indicating similar results with those obtained from comparing the means of sub-populations formed by the presence or absence of rosette shape. Conversely, no significant correlation was observed between hairy glumes and other characters. This also confirmed the results obtained by comparing sub-populations formed by the presence or absence of hairy glumes. On the other hand, no significant correlation was found between the polymorphic band amplified using OPG12 and days to maturity and between polymorphic band amplified using OPM35 and DH. These results approved that relying on simple correlation coefficients is not sufficient when studying any possible link between molecular markers and other quantitative traits. Mean while, the results of t-test in which the mean of the sub-populations having the bands amplified by OPG12 and OPM35 were significantly different for days to maturity and days to heading (Tables 5 and 4), respectively from those lacking the bands. This indicates the suitability of t-test for looking any possible link between molecular markers and quantitative traits. GY negatively correlated with day to heading (-0.40**) and rosette growth (-0.37**) and it was positively correlated with harvest index (0.66**). Moghaddam et al., (1998) also reported a positive correlation between HI and grain yield. HI itself correlated negatively with days to heading, days to maturity and rosette growth justifying positive correlation between harvest index and grain weight.

Table 8. A matrix of simple correlation coefficients for the markers and the quantitative traits under study

	SL	GW/P	HG	DH	DM	RS	HI	OPM35	OPG12
SL	1								
GW/P	.21	1							
HG	-.06	-.07	1						
DH	-.04	-.40**	.01	1					
DM	-.11	-.21	.07	.46**	1				
RS	-.07	-.37**	-.02	.82**	.40**	1			
HI	.04	.66**	.01	-.42**	-.34**	-.35**	1		
OPM35	.12	.20	.16	-.28	.13	-.19	.12	1	
OPG12	-.11	-.09	.13	-.09	-.31	-.12	.08	..18	1

*and **: significant at 5% and 1% level of probability, respectively.
SL: spikelet length, GW/P: grain weight per plant, HG: hairy glume, DH: days to heading, DM: days to maturity, RS: rosette shape of growth for a period of 30 days from planting date, HI: harvest index

Discussion

Molecular markers

In the present study, a simple approach was used to cluster F_2 population for which no similar method was observed in the literature. In this method, single markers were used to differentiate individuals within the F_2 population. Meanwhile, almost all of the investigators have used multivariate cluster analysis to classify individuals into different groups. In this regard, in a study carried out by Liu et al. (1999), fifty-four RAPD markers generated by six primers were used to study their potential power in differentiating parents with different characteristics and predicting the yield performance of hybrids produced from these parents. Based on the genetic distance matrix, they were able to divide the genotypes under consideration into four groups. Group I was characterized by more grains per spike, group II by heavy grains and group III by more spikes per unit area and short plants; group IV was similar to group III but had a much higher biomass yield and grain yield. Based on the above results, they concluded that it is possible to differentiate wheat lines with different performances using RAPD markers. The latter method was not a case for present study because there were only two polymorphic markers and so not enough to form genetic distance matrix. The molecular marker amplified by OPM35 was able to differentiate F_2 individuals for heading date. This character is one of the most important traits for wheat performance and improvement because it affects the adaptability of the crop to environmental conditions including water-stress. (Law et al.. 1998; Law, 1998). The genetic control of heading date is complex and many genes are responsible for the character including photoperiod genes, vernalisation genes and earliness per se genes (Snape, 1987). This complexity then implies that direct selection for the character is less likely to be successful and that using molecular marker is likely to result in a higher gain of selection. To sum up, the results of the present study revealed that using single molecular markers to screen for quantitative traits is possible and the experiments carrying with molecular markers could be analyzed even only one polymorphic molecular bond is amplified. However investigating the quantitative characters in the classified groups at the F_3 generation would reveal further information useful for crucial speculation regarding the links between the phenotypic markers and quantitative characters. A study is under way to investigate the classified sub-populations at the F_3 generation

Rosette shape

Vernalisation requirement is the sensitivity of plants to cold treatment for accelerating spike primordia development (Kato et al., 2001). Semi-winter varieties such as the variety used in the present study (FM36) remain in a rosette shape to a period up to 30 days planting in spring where they do not meet vernalisation requirements (Kainth 1994). At these situations, vernalisation sensitive genotypes reach the stage of stem elongation with delays. This behavior of growth makes it possible to identify the response of the plants when they are grown without any artificial cold treatments. Thus using a rosette shape as a morphological marker in order to differentiate the F_2 individuals in fact reflects the effects of vernalisation response. Vernalisation responses of wheat cultivars play an important role in adaptation of these cultivars to environmental conditions. Although the effect of vernalisation is generally to promote earlier floral development (Worland, 1996), the effect of vernalisation on the heading date and morphological characters is dependent on genotype and environment (Kato et al., 2001). Vernalisation requirement is related to drought and heat tolerance in the regions in which plants are to be grown under limited water supply or high temperatures. In this situation, if temperature is not low enough to satisfy vernalisation, plants produce massive vegetative tissues in the spring and consequently use a high proportion of water supply before starting the reproductive stage. Even if water supply is not limited, lack of vernalisation postpones anthesis and causes damages to the plants due to high temperatures during the grain-filling period. Such a phenomenon happened in the present study caused a significant reduction in grain weight of the genotypes sensitive to vernalisation.

Hairy glumes

Hairy glume is under the control of a single dominant gene (McIntosh et al., 1998) and is a very clear character that is easily notable in segregating generations. In the present experiment this characters was not able to differentiate between individuals for the quantitative traits. This result is similar to results obtained by Lage et al., (2003) reporting the lines having hairy glumes were not significantly different for harvest index, grain weight, spike length and other agronomic traits from those having hairless waxy glumes. However, it is associated with powdery mildew resistance in a Korean wheat variety called Suwon 92 (XU et al., 2006).

Comparing between correlation analysis and t-test

Comparing the results obtained by t-test with those by correlation analysis revealed that the two statistical analyses were not performed in a similar way for all the characters. For instance, t-test analysis revealed a significant difference between the sub-population having the polymorphic band amplified by using OPM35 and that lacking the band for days to heading. On the other hand, correlation coefficient between the band and days to heading was not significant. These results imply that correlation analysis does not support the finding of t-test. The reason for this inconsistency may relate to the assumption needed for parametric analyses such as correlation coefficient. When using distinct numeric 0 and 1 as one set of data, the statistical assumption for correlation analysis (means normality distribution) (Steel and Torrie 1976) is not achieved. Thus, it seems that clustering individuals into two groups based on the absence or presence of a particular marker and then performing t-test to compare the means of the two groups having homogenous quantitative data is more compatible with theory of statistics and then possibly more reliable than calculating correlation between two heterogeneous sets of data.

Materials and methods

Plant materials

One hundred and eighty plants from an F_2 population, derived from crosses between the Pakistani variety Faisalabad and the line FM36, along with 20 plants from each parent were used in the present study. Faisalabad is a spring variety having a strong gene for vernalisation insensitivity indicating no requirement to vernalisation and completes its growth period earlier. FM36 is a line derived from improved generations of a cross between varieties Mexicani and Falchetto (Mohammady 2002). The line FM36 needs vernalisation to accelerate it reproductive growths and without vernalisation, it will remain vegetative in a rosette shape for a period up to 30 days after planting. This line is sensitive to artificial vernalisation and has facultative growth habit and after 30 days, it starts stem elongation stage (Haidar 1997, Kainth 1994). Faisalabad has non-hairy glumes while FM36 has hairy glumes.

Experimental conditions

The parents and F_2 individuals were planted in an evenly field station in Shahrekord region, south west of Iran (50° 51' E, 32° 19' N) in April 2006 when the temperature was enough high to allow identification of vernalisation sensitive genotypes. The distances between plants on the row and between the rows were 10 and 30 cm, respectively. Thirty days after planting, the genotypes sensitive to vernalisation were identified based on their rosette growth. The number of genotypes sensitive to vernalisation (but facultative growth habit) and those insensitive to vernalisation (having spring growth habit) were also recorded. Heading date was recorded as the number of days from planting to heading stage in which the spikes fully emerged. Spike length was measured in cm and days to physiological maturity were calculated. Genotypes having hairy glumes and those lacking hairy glumes were also identified. At harvesting, the above ground biomass of each plant was dried in an incubator at 80 C^o for 24 hours and was weighted to estimate the aboveground dry matter production. The grains of each plant were threshed out separately and the amount of grain weight per plant was recorded. Harvest index was calculated, as a ratio of grain weight to aboveground biomass, for each plant. Genomic DNA was isolated from fresh young leaves of the parents and the F_2 individuals using the modified CTAB method (Murry and Thompson 1980). RAPD analysis was performed using 30 primers. Polymerase chain reaction was performed according to Table 1. Amplified products were separated on 1.2% agarose gels in 5X TBE. Gels were stained with ethidium bromide and visualized under UV light.

Statistical Analysis

The RAPD profiles were analyzed based on the presence (1) or absence (0) of individual RAPD bands. Two molecular bands, amplified by primers OPM35 and OPG12, respectively, indicated polymorphism in parents as well as in F_2 population (Fig 1). Knowing these results, the F_2 individuals were classified into 2 groups based on presence or absence of the bands amplified by the primers or absence or presence of qualitative traits (including hairy glumes and rosette shape), separately. With this explanation, it is clear that 4 visual grouping systems were provided. In each case, the quantitative characters of individuals in the two groups were then recorded and a t-test was performed to see whether the differences observed between the means of the two groups are significant for the quantitative traits. In addition, Pearson correlation analysis was also performed to simply identify possible phenotypic link between the characters and to compare the results obtained from correlation analysis with those obtained from t-test. For performing correlation analysis between quantitative and qualitative characters, the latter characters were coded as 0 and 1 (having the character 1 and lacking the character 0).

Conclusion

The results of the present study revealed that using single molecular markers to screen for earliness is possible and the experiments carrying with molecular markers could be analyzed even only one polymorphic molecular bond is amplified. In addition, it was revealed that using RAPD markers and rosette shape was successful in classification of F_2 population to earliness groups while using hairy glume failed to differentiate between F_2 individuals for earliness.

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