Identification of AFLP markers associated with flowering time and ornamental traits in *Chrysanthemum*

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Abstract
Flowering period and longevity play important roles in determining the quality of commercial flowers. Marker-trait associations for eight flowering and 12 ornamental traits have been studied using a GLM and MLM analysis with a set of 2099 AFLP polymorphic markers in Chrysanthemum. The GLM model identified 453 markers for phenotypic traits whereas the MLM association analysis model revealed a total of 197 significant marker-trait associations for the phenotypic traits. The strongest association was detected between AFLP markers with a bud diameter trait, which explained 68% of the variation. Among several polymorphic bands, 14 markers were associated with senescence, 10 with flower diameter and eight with stem length. This approach also led to the identification of seven markers with significant association to full bloom. Therefore, these markers can be used for the genetic improvement of the ornamental value of Chrysanthemum after further confirmation. The analysis of the results revealed a number of markers co-associated with different correlated phenotypic traits. The results revealed informative markers that have shown a significant correlation with several traits which could be useful for breeding programs and other analyses associated to future studies of Chrysanthemum.

Key words: Association analysis, *Chrysanthemum morifolium,* Correlation, Phenotypic traits, Senescence.

INTRODUCTION

Chrysanthemum (*Chrysanthemum morifolium*) is a short-day (SD) herbaceous perennial and widely cultivated plant for ornamental purposes such as cut flowers, potted and ground-cover across the world (Sun *et al.*, 2010; Nakano *et al.*, 2013). Chrysanthemum is adapted to a temperate climate and its optimal growth temperature lies in the range of 18-21 °C (Fang *et al.*, 2009). Chrysanthemums are usually harvested when the blossoms are about one-third open (Nishi *et al.*, 2009). The ornamental value and vase life of a spray cut Chrysanthemum usually drop with an increase in the quantity of pollen dispersal at the flowering stage (Wang *et al.*, 2014). Therefore, Wang *et al.* (2014) suggested developing new cultivars with less-dispersed or non-dispersed pollen through breeding programmes. For the purpose of improving traits, understanding the inheritance pattern is necessary (Zhang *et al.*, 2011). In agricultural species, the recognition of varieties and breeding lines is very important (Martin *et al.*, 2002). Traditionally, the identification of ornamental plant cultivars has been based on phenotypic traits (colour of the inflorescence, petal shape) but this method needs the plants to be seen in flower and at a complete growth cycle. In parallel, molecular marker technologies were developed to allow these analyses to be based on DNA information and to give clear and more direct information of the genetic polymorphisms of plants, so that only small samples of leaves can be enough for analysis in the early cutting stage (Martin *et al.*, 2002; Teixeira da Silva, 2004). One of the limiting factors in the genomic analysis of many plant species is that the

genomic studies between experimental populations are the result of crossing two parents (Achleitner *et al.*, 2008). Traditional QTL mapping is very costly, has poor resolution with the evaluation of only a few alleles and requires a longer research time period (Hedrick *et al.*, 1987; Devlin and Risch, 1995). Thus, in many QTL the reports are limited to a specific genetic background and the success of their application is limited (Achleitner *et al.*, 2008). To solve this problem, researchers are currently using association analysis as an alternative approach to detect genes and QTLs from random sets including genotypes with mixed genetic backgrounds (Rostoks *et al.*, 2006; Breseghello and Sorrells, 2006). In recent years, genome-wide association studies (GWAS) have been developed for plants because the resolution of their marker-trait associations is superior to that of conventional QTL analyses (Gawenda *et al.*, 2012) and it has also appeared as a tool to resolve complex trait variations in plants at the population level (Nordborg and Tavare, 2002). Furthermore, association studies reduced research time and analysed greater numbers of allele (Yu and Buckler, 2006). Association analysis as a strategy and a promising approach has been successfully applied in horticulture crops such as pak-choi (Yu *et al.*, 2010), jasmine (Chayanika, 2012), and Phalaenopsis orchids (Gawenda *et al.*, 2012). The first step to genetically improve Chrysanthemum as an economic ornamental crop is to identify genetic regulation of key ornamental and horticultural traits such as flower size, flower type, stem length, leaf shape and flowering time. To achieve this goal, it is necessary to identify all the genes underlying the ornamental characteristics and markers that can be used to select these traits through marker-assisted selection. In spite of critical needs for understanding the population genetics of Chrysanthemum, little information is available on a genetic linkage map, QTL (Zhang *et al.*, 2010; Zhang *et al.*, 2012) and the marker-trait associations of Chrysanthemum (Zhang *et al.*, 2011). Zhang *et al.* (2011) identified SRAP markers associated with initial blooming time and the duration of flowering. However, there has still been no report produced on other phenotypic and flowering traits. Most genetic studies on Chrysanthemum have focused on characterizing genetic diversity using ornamental traits of inflorescence and molecular markers (Martin *et al.*, 2002; Shao *et al.*, 2010; Zhang *et al.*, 2011). Despite the economic importance of Chrysanthemum production in Iran, no major study has been carried out to identify and estimate the genetic structure of our breeding materials. In previous studies, we have reported the first study of a population structure of Chrysanthemum genotypes of Iran (Roein *et al.*, 2014). Our previous research focused on the genetic diversity and population structure of Chrysanthemum. We clustered genotypes and identified four major subpopulations. However, there is no information available about the association between the traits and the markers. Therefore, the objective of the present study was to identify associated AFLP markers with flowering time and ornamental traits in Chrysanthemum using the association analysis approach for potential application in breeding programmes on Chrysanthemum. This report is, to our knowledge, the first association study carried out using a collection of Chrysanthemum genotypes.

MATERIALS AND METHODS

Plant material

Forty-eight genotypes of Chrysanthemum were chosen for this study. These genotypes are representative samples of the gene pool currently used in the breeding programmes of the National Research Centre of Ornamental Plants, Mahallat, Iran (Supplementary Table 1). All the genotypes were grown in the greenhouse conditions, where the growth temperature was set at 22 ± 3 °C. The genotypes were potted in four replications at the University of Guilan.

Evaluation of phenotypic traits

The morphology of the flowers and leaves was evaluated for the germplasm. A total of 20 phenotypic traits (ornamental and eight flowering parameters) were recorded for flowering. A summary of the analysed traits and further details regarding the measurement and calculation of traits are described in Table 1. Different stages of flowering in Chrysanthemum genotypes are presented in Figure 2. The Pearson correlation coefficients were calculated for all pairs of variables. Analyses were carried out with SPSS 21.0 software (IBM Corp, 2012).

Marker analysis

Genomic DNA was extracted from young leaves of greenhouse grown plants using the CTAB method (SaghaiMaroof *et al.*, 1984). DNA quality was visually examined using 1% (w/v) agarose gel stained with ethidium bromide. AFLP markers were generated according to the method used by Vos *et al.* (1995) using *Mse*I and *Eco*RI (Fermentas) restriction enzymes. In total, 25 AFLP primer combinations that contained two to three selective nucleotides in the 3' end of each primer were performed. The details of the *Eco*RI and *Mse*I primers are given in supplementary Table 2. AFLP fragments were separated on a polyacrylamide gel (6%). Gel images were scored visually and

Table 1. Phenotypic traits recorded for the chrysanthemum genotypes under study.

Trait type	Trait	Code	Trait description	Unit
Ornamental	Leaf length	LL	Length of leaves at full bloom stage	cm
	Leaf width	LW	Width of leaves at full bloom stage	cm
	Pedicel length	PedL	Length of pedicel at full bloom stage	cm
	Stem length	SL	Length of stem at full bloom stage	cm
	Petiole length	PetL	Length of petiole in primary flowers	cm
	Ray floret number	RFN	Number of ray floret in primary flowers	Number
	Tubular floret number	TFN	Number of tubular floret in primary flowers	Number
	Ray floret length	RFL	Length of ray floret in primary flowers	cm
	Ray floret width	RFW	Width of ray floret in primary flowers	cm
	Flower bud diameter	FBD	Diameter of first flower bud	cm
	Flower diameter	FD	Diameter of first flower	cm
	Number of flower per plant	NF/P	Total number of flower	Number
Flowering time	Days to visible flower bud	VFB	Days from transplantation to observe of flower bud	Days
	Days to color shown of flower bud	CSFB	Days from observe of flower bud to color shown	Days
	Days to complete opening of ray floret	CORF	Days from color shown to complete opening of ray floret of primary flowers	Days
	Days to onset opening of tubular floret	OTF	Days from complete opening of ray floret to onset opening of tubular floret	Days
	Days to complete opening of tubular floret	COTF	Days from onset opening of tubular floret to complete opening of tubular floret	Days
	Full bloom	FB	Days from opening of fist flower to opening of 80 percent of the flowers in the plant	Days
	Senescence of first flower	SPF	Days from complete opening of ray floret of primary flowers to wilting of secondary ray floret	Days
	longevity of post-production	LPP	Days from complete opening of ray floret of primary flowers to senescence of 15 % of flowers per plant	Days

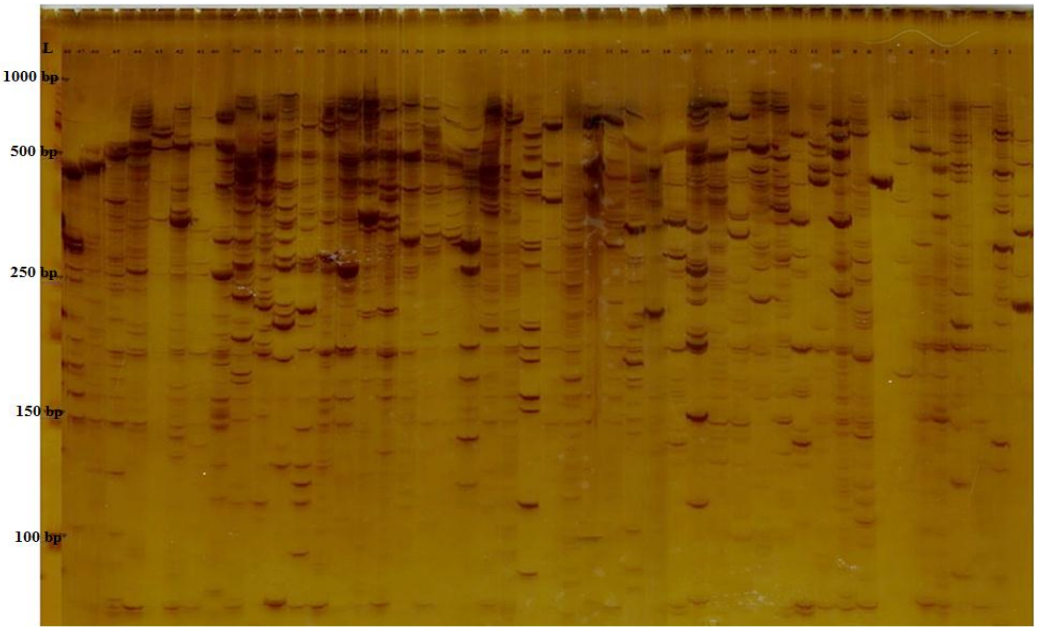

Figure 1. AFLP banding pattern of 48 genotypes of Chrysanthemum obtained by the M-CAG/E-AAC primer.

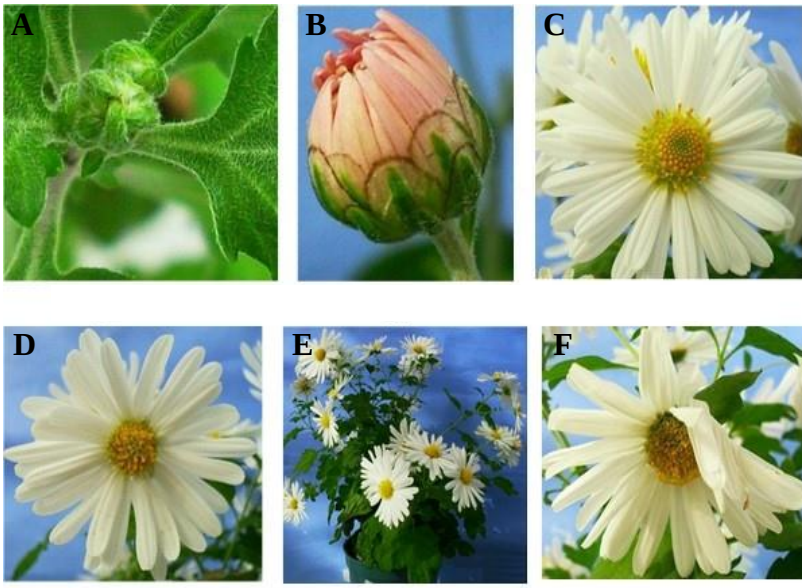

Figure 2. Flowering stages of Chrysanthemum (Gita genotype). **A:** days to visible flower bud, **B:** days to color shown of flower bud, **C:** days to onset opening of tubular floret, **D:** days to complete opening of tubular floret, **E:** full bloom, **F:** senescence of first flower.

polymorphic bands were recorded as present or absent. Monomorphic AFLP bands were not included in the statistical analysis (Figure 3).

Statistical analyses

The population structure was analysed using the software program STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) to estimate the number of genetically distinct populations. Iterations were performed 10,000 times using a burn-in length of 100,000 MCMC (Markov Chain Monte Carlo) with the admixture and related frequency model. Five independent simulations were performed for each k (the number of populations), ranging from 1to 10. To estimate the appropriate value for K, delta K was used, in line with the method described by Evanno *et al.* (2005). The population structure matrix (Q) that has the membership coefficients of an individual in a sub population was identified by running the STRUCTURE program at K= 4. The relative kinship matrix (K matrix) was obtained using the program TASSEL 3.1 (Bradbury *et al.*, 2007). The mean phenotypic values were used for the association analysis. Using the software TASSEL, version 3.1, two methods were used to test for associations between AFLP markers and phenotypic traits. First, a general linear model (GLM) was tested to identify AFLP markers effects on phenotypic traits. This analysis considers the population structure detected by STRUCTURE (Q matrix) as co-factors. Second, the Mixed Linear Model (MLM) was used as suggested by Yu *et al.* (2006). The Q+K, MLM method that combines data from both Q and K was run in TASSEL 3.1. Both models were tested for each of the 2099 AFLP markers. Finally, to eliminate possible spurious associations, we focused on significant associations obtained using the MLM approach of Yu *et al.* (2006).

RESULTS

Phenotypic evaluation and correlations

The Pearson's correlation coefficients between pairs of traits are shown in Table 2. The length and width of leaves were highly correlated with each other. Also, correlations were found between the length and width of the ray floret, stem length and pedicel length. In contrast, a significant negative correlation was found between flower diameter and the number of flowers per plant (Table 2). This suggests that when the flower is small, the number of total flowers per plant increases. Furthermore, a significant negative correlation was obtained between days to colour of the flower bud and the senescence of the first flower and the longevity of post-production.

Association analysis

A total of 25 AFLP primer combinations produced 2099 AFLP polymorphic bands for the 48 individuals

Table 2. Pearson's correlation coefficients between pairs of phenotypic traits studied in the chrysanthemum germplasm.

Trait	LL	LW	PedL	SL	PetL	RFN	TFN	RFL	RFW	FBD	FD	NF/P	VFB	CSFB	CORF	OTF	COTF	FB	SPF
LW	0.90**	1																	
PedL	0.63**	0.51**	1																
SL	0.56**	0.56**	0.38*	1															
PetL	ns	ns	ns	ns	1														
RFN	ns	ns	ns	ns	0.37*	1													
TFN	0.31*	0.39**	ns	ns	ns	-0.31*	1												
RFL	0.40**	0.37*	ns	ns	ns	0.30*	ns	1											
RFW	0.44**	0.46**	ns	ns	ns	ns	0.35*	ns	1										
FBD	0.54**	0.52**	ns	ns	ns	0.31*	0.37*	0.38*	0.40**	1									
FD	0.37*	0.34*	ns	ns	ns	ns	ns	0.60**	0.30*	-0.40**	1								
NF/P	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.42**	-0.48**	1							
VFB	ns	ns	0.57**	ns	ns	ns	ns	ns	ns	ns	ns	ns	1						
CSFB	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.30*	ns	1					
CORF	ns	ns	ns	ns	ns	0.36*	ns	ns	ns	ns	ns	ns	ns	ns	1				
OTF	ns	ns	ns	ns	ns	0.41**	ns	ns	ns	ns	ns	ns	ns	ns	ns	1			
COTF	ns	ns	ns	ns	ns	ns	0.37*	ns	ns	ns	ns	ns	ns	ns	ns	ns	1		
FB	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.37*	1	
SPF	ns	ns	ns	0.40**	ns	ns	ns	ns	0.32*	ns	ns	ns	-0.41**	ns	ns	0.44**	ns	ns	1
LPP	ns	ns	ns	ns	ns	ns	ns	ns	0.31*	ns	ns	ns	-0.50**	ns	ns	0.31*	ns	0.48**	0.67**

ns, *, **: not significant, significant difference at P<0.05, P<0.01 (2-tailed).

LL: Leaf lengh, LW: Leaf width, PedL: Pedical length, SL: Stem length, PetL: Petiole length, RFN: Ray floret number, TFN: Tubular floret number, RFL: Ray floret length, RFW: Ray floret width, FBD: Flower bud diameter, FD: Flower diameter, NF/P: Number of flower per plant, VFB: Days to visible flower bud, CSFB: Days to color shown of flower bud, CORF: Days to complete opening of ray floret, OTF: Days to onset opening of tubular floret, COTF: Days to complete of tubular floret, FB: Full bloom, SPF: Senescence of first flower, LPP: Longevity of post-production.

Table 3. Number of markers associated with phenotypic traits of chrysanthemum using GLM and MLM models.

Trait	Number of markers associated with trait	
	GLM (Q)	MLM (Q+K)
Leaf length	34	11
Leaf width	27	12
Pedicel length	20	9
Stem length	24	8
Petiole length	24	8
Ray floret number	8	2
Tubular floret number	40	6
Ray floret length	10	5
Ray floret width	15	10
Flower bud diameter	19	15
Flower diameter	35	10
Number of flower per plant	13	6
Days to visible flower bud	31	20
Days to color shown of flower bud	20	9
Days to complete opening of ray floret	25	7
Days to onset opening of tubular floret	17	13
Days to complete opening of tubular floret	27	15
Full bloom	17	7
Senescence of first flower	28	14
longevity of pot production	19	10
Total	453	197

of Chrysanthemum (Roein *et al.*, 2014). In this study, the association analysis of 2099 molecular markers (AFLP) with 20 flowering and ornamental related traits was evaluated using GLM and MLM procedures. Significant associations were observed between markers and phenotypic traits for two of the tested models (Table 3). The results of the association analysis, using TASSEL software, showed the number of significant associations was reduced from 453 in the GLM model to 197 in the MLM model. We focused on the significant associations using the MLM model, since they are more reliable. This model is useful for reducing and correcting false positive associations (Bradbury *et al.*, 2007). Because this approach considers both the kinship matrix and the population structure Q matrix in the marker-trait association test. According to the Q + K, MLM method, based on the 2099 AFLP marker fragments we found 197 markers associated with at least one of the 20 phenotypic traits (Table 3). Markers associated significantly ($p<0.01$) with r^2 value of 17% or more, were selected. The results of the association analysis revealed 11 and 12 markers for the length and width of the leaf, respectively. M-CAG/E-AGA-56 marker was significantly associated with the length of leaf ($p=8.69\times10^{-04}$) and explained 30% of the total variation. The nine AFLP markers, associated with traits of leaf length and leaf width, were similar. Eight molecular markers have shown an association with stem length. Moreover, M-CAC/E-AAG-10 marker was associated with stem length and was also significantly associated with leaf length and leaf width. According to the results, only two markers (M-CAA/E-AAC-28 and M-CAC/E-AGA-29) were associated with the ray floret number which explained 18 and 20% of variation. In contrast, we were also able to identify 20 markers associated with days to visible flower bud. The strongest association was detected between the AFLP markers of M-CAC/E-AAC39 and M-CTT/E-ACA-1, with the bud diameter, explaining 68% of variation with a p-value of 1.52×10^{-05} and 2.66×10^{-06}, respectively. Results indicated that nine AFLP markers were associated with days to colour appearance of flower bud. In particular, seven markers were strongly (30-39% of variation) associated with the traits which were also significantly ($p=4.51\times10^{-04}$) associated with the onset of tubular floret opening (43-45% of variation). The minimum p value for the onset of tubular floret opening was 4.55×10^{-04}. Fourteen associations were observed between AFLP markers and the senescence of the first flower, while 10 markers were responsible for associations with the longevity of post-production (Table 3). We also found that some markers were associated simultaneously with two or more traits (Table 4). MCAG/E-AAG-72 marker was associated with days to visible flower bud ($p=0.004$), days to colour of the flower bud ($p=0.002$) and the onset of tubular floret opening ($p=4.55\times10^{-04}$), while M-CAA/E-AGA-54 was associated with leaf length ($p=0.009$), leaf width ($p=0.008$) and the width of ray floret ($p=0.0039$).

DISCUSSION

Flowering time is a very important developmental and essential determining trait for adaptation during crop domestication which is affected by environmental stimuli such as photoperiod. Angiolini *et al.* (2015) reported that morphological variation is associated with geographical variables, soil chemistry and habitat types. Furthermore, flower longevity is the most important factor for the explanation of ornamental value. Therefore, recognition of correlation data can be useful for plant breeders to anticipate the relatedness of traits and perform indirect selection for other traits (Carter *et al.*, 2011; Portis *et al.*, 2014). Moreover, correlation analysis indicated that a group of tightly correlated

Table 4. P values for AFLP markers that associated with multiple phenotypic traits.

Primer	LL	LW	PedL	SL	PetL	RFL	RFW	FD	NF/P	VFB	CSFB	OTF	SPF	LPP
M-CAC/E-AAG-10	0.005395	0.001068		0.00152										
M-CAG/E-ACA-23	0.007919	0.003726												
M-CAG/E-ACA-24	0.005946	0.007976	0.004212											
M-CAG/E-ACA-29	0.008018	0.008248						0.004641						
M-CAG/E-AGA-56	8.69E-04	0.003009												
M-CAA/E-AAG-40	0.009143	0.004693												
M-CAA/E-AGA-54	0.009225	0.008383					0.003911							
M-CTG/E-ACA-16	0.006335	0.007052												
M-CTG/E-AGA-92	0.009786	0.008815												
M-CAC/E-AAC-49			0.001347							1.90E-04				
M-CAG/E-AAC-8			0.005498							2.50E-04				
M-CTT/E-AAC-1			0.008436							0.002258				0.009103
M-CAC/E-AGA-89				0.008856	0.005998									
M-CTG/E-AAG-3					0.008848									0.001611
M-CTG/E-ACC-45					0.005681		0.002485							
M-CTG/E-AA-29						0.002678		0.005323						
M-CTG/E-ACC-16							0.003668						0.006052	0.008156
M-CAC/E-AGA-96								0.005971		0.005744				
M-CAC/E-ACC-47								0.009489	0.005381					
M-CAG/E-AAG-72										0.004096	0.002205	4.55E-04		
M-CAC/E-AGA-22											0.002231	3.51E-04		
M-CAC/E-ACC-80											0.001396	4.54E-04		
M-CAC/E-ACG-92											8.92E-04	3.40E-04		
M-CAG/E-ACA-53											0.002171	4.51E-04		
M-CAG/E-AGA-3											0.001966	4.19E-04		
M-CAA/E\-AAC-55											0.002147	4.45E-04		
M-CAC/E-AAG-95													0.00873	0.008642

LL: Leaf length, LW: Leaf width, PedL: Pedical length, SL: Stem length, PetL: Petiole length, RFL: Ray floret length, RFW: Ray floret width, FD: Flower diameter, NF/P: Number of flower per plant, VFB: Days to visible flower bud, CSFB: Days to color shown of flower bud, OTF: Days to onset opening of tubular floret, SPF: Senescence of first flower, LPP: Longevity of post-production.

traits may share a common genetic basis (Kim and Xing, 2009). A close correlation between phenotypic traits was observed. It is also interesting to note that flower longevity was not affected by the number of flowers per plant, but was negatively correlated (r=-0.41) with days to the visible flower bud, a very important trait for the beginning of the reproductive phase. Correlation among the flowering parameters studied showed that the number of flowers per plant had the highest and negatively significant correlation with flower diameter. A similar conclusion was also reached by Misra *et al.* (2013). One of the most practical applications of DNA-based markers in breeding is the ability to select phenotypic traits and markers closely linked to genes controlling these traits (Forcada *et al.*, 2013). This is the first time that associations between AFLP markers and 20 phenotypic traits in 48 Chrysanthemum genotypes have been analysed. A comparison of the GLM and MLM models showed that the MLM model minimizes the possibility of false positive associations between marker and the phenotype (Bradbury *et al.*, 2007). Because of this, only the results from the MLM model were discussed in this study. The main findings from this study showed significant associations between several traits and markers. We found 197 marker-trait associations for 20 phenotypic traits using the MLM method ranging from two to 20 associations. In Chrysanthemum, flower size as a breeding characteristic, is highly important. Our study identified 10 markers associated with flower diameter. In contrast, Chayanika (2012) found two AFLP markers associated with the flower diameter of jasmine. Gawenda *et al.* (2012) reported two AFLP markers associated with the flower size of Phalaenopsis orchids and identified 10 markers for stem length. This is in line with our findings as we found eight markers associated with the stem length trait. Yagi *et al.* (2014) mapped the D85 locus, controlling the flower type of a carnation using a SSR and it was suggested as being potentially useful for the marker-assisted breeding of carnations. The highest r^2 value of 68% was found between the AFLP markers M-CTT/E-ACA-1 and M-CAC/E-AAC-39 with the bud diameter. Similar results were reported by Chayanika (2012) who found a similar significant association (68%) between AFLP markers and flower stalk length. Understanding the mechanisms of flower senescence is useful for improving postharvest flower quality and longevity. The application of association analysis for senescence may facilitate the improvement of flower longevity in Chrysanthemum. In our study, 14 AFLP markers were associated with senescence. The lowest P-value of markers associated with senescence occurred in M-CAG/E-AAC-9 (P=0.0012, r^2=28%). The opening of tubular floret causes the release of pollen in Chrysanthemum (Figure 2). The process is an undesirable factor during its flowering stage and can significantly reduce its ornamental value and quickly shorten its vase life. The results of this study showed that the days to the onset of the opening of tubular floret are associated with 13 AFLP markers. Some of the AFLP markers showed a significant P value for more than one trait. The length and width of the leaf appeared to be associated with the same set of markers. Markers that provided the highest p-values of the length of leaf also provided the highest p-values of the width of leaf. Although senescence and stem length were positively correlated with r=0.40, no common significant markers were detected for these two traits. It should be noted that we found a significant correlation between various phenotypic traits. For example, flower diameter showed a significant positive correlation with the length and width of the leaf and length and the width of ray floret. This correlation was also evident in shared associated markers for these traits. The M-CAG/E-ACA-29 marker correlated with flower diameter and with traits correlated to those, such as length and width of leaf. It is noteworthy that, the days to the onset of the opening of the tubular floret shared seven markers with days to the colour of the flower bud. On the other hand, this study identified two markers associated with ray floret number, whereas these were not associated with any other traits. It is possible that the correlation between traits and the association between traits and markers suggest pleiotropy in the genomic region. This may also reveal QTLs closely linked with different traits and lead to a single marker showing an association with multiple traits, correlated with such traits (Rakshit *et al.*, 2010). Based on the traits affected, there are a number of markers that we consider to be the most interesting candidates for further work. Moreover, informative markers such as M-CTG/E-ACC-16, M-CAC/E-AAG-95, M-CAG/E-ACA-29 and M-CAC/E-AAG-10 shown to have significant correlations with several traits, which can be used for breeding programmes and other analyses associated to future studies of Chrysanthemum. Several studies have reported associations between single markers and several traits (Mazzucato *et al.*, 2008; Yan *et al.*, 2009; Gawenda *et al.*, 2012; Saïdou *et al.*, 2014). This result might be caused by the pleiotropic effects of linked genomic regions or the genetic reasons for correlation among traits (Koyama *et al.*, 2001; Gawenda *et al.*, 2012; Zhao *et al.*, 2013). Zhang *et al.* (2011) identified SRAP markers associated with initial blooming time (10 markers) and the duration of flowering (12 markers) that explain, respectively, 46 and 54% of the variation.

The association study by Zhao *et al.* (2007) detected eleven markers associated with the days to flowering of *Brassica rapa*. Mannai *et al.* (2011) identified a large number of markers associated with flowering time of Sorghum with different levels of significance. In marker-assisted breeding, one marker is co-associated with multiple traits which are correlated and it can be used to identify all these traits for selection. This clearly improves breeding efficiency and increases the chances of a trait appearing alongside traits when strongly correlated with them (Yan *et al.*, 2009). In this study the correlation between traits and associations between traits and markers further confirmed the resulting association analysis of the flowering parameters and ornamental characteristics. Therefore, the associations determined in the present study would be useful for the deployment of marker assisted selection (MAS) in Chrysanthemum breeding programmes. Although, further research is required to confirm these associations either with additional markers or populations with a different genetic background. Preliminary research was conducted in this study, therefore, further research is necessary in this field.

CONCLUSION

To come to a conclusion, for identifying markers associated with flowering and ornamental traits, we performed an association analysis on Chrysanthemum genotypes with 2099 AFLP markers. The results of our study demonstrate a significant potential of an association analysis of phenotypic traits, related to flowering parameters and senescence, in Chrysanthemum with AFLP markers. To the best of our knowledge, this work is the first approach to conducting an association analysis study with ornamental traits in Chrysanthemum. The markers with the strongest effects in our study provide ideal candidates for further study and are useful in practical breeding programmes for developing new cultivars of Chrysanthemum.

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REFERENCES

Achleitner A., Tinker N. A., Zechner E., and Buerstmayr H. (2008). Genetic diversity among oat varieties of worldwide origin and associations of AFLP markers with quantitative traits. *Theoretical and Applied Genetics*, 117: 1041–1053.

Angiolini C., Bonari G., Frignani F., Iiriti G., Nannoni F., Protano G., and Landi M. (2015). Ecological patterns of morphological variation in italian populations of *Romulea bulbocodium* (Iridaceae). *Flora*, 214: 1–10.

Bradbury P. J., Zhang Z., Kron D. E., Casstevens T. M., Ramdoss Y., and Buckler E. S. (2007). TASSEL: Software for association mapping of complex traits in diverse samples. *Bioinformatics*, 23: 2633–2635.

Breseghello F., and Sorrells M. E. (2006). Association mapping of kernel size and milling quality in wheat (*Triticum aestivum* L.) cultivars. *Genetics*, 172: 1165–1177.

Carter A. H., Garland-Campbell K., and Kidwell K. K. (2011). Genetic mapping of quantitative trait loci associated with important agronomic traits in the spring wheat (*Triticum aestivum* L.) cross 'Louise' 9 'Penawawa'. *Crop Science*, 51: 84–95.

Chayanika S. (2012). Morphological and DNA marker - based genetic diversity assessment and tagging QTLs controlling economic traits in jasmine (*Jasminum* spp.). *University of Agricultural Sciences*, p.167.

Devlin B., and Risch N. (1995). A comparison of linkage disequilibrium measures for fine-scale mapping. *Genomics*, 29: 311–322.

Evanno G., Regnaut S., and Goudet J. (2005). Detecting the number of clusters of individuals using the software structure: A simulation study. *Molecular Ecology*, 14: 2611–2620.

Fang W. M., Guo W. M., and Chen J. Y. (2009). Effects of grafting on the improvement of heat tolerance and antioxidant abilities in leaves of chrysanthemum. *Acta Horticulturae Sinica*, 36: 1327–1332.

Forcada C. F., Igartua N. O. E., Moreno M. A., and Gogorcena Y. (2013). Population structure and marker-trait associations for pomological traits in peach and nectarine cultivars. *Tree Genetics and Genomes*, 9: 331–349.

Gawenda I., Schroder-Lorenz A., and Debener T. (2012). Markers for ornamental traits in phalaenopsis orchids: Population structure, linkage disequilibrium and association mapping. *Molecular Breeding*, 30: 305–316.

Hedrick P. W. (1987). Gametic disequilibrium measures: Proceed with caution. *Genetics*, 117: 331–341.

IBM Corp. Released. 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.

Kikuchi R., and Handa H. (2009). Photoperiodic control of flowering in barley. *Breeding Science*, 59: 546–552.

Kim S., and Xing E. P. (2009). Statistical estimation of correlated genome associations to a quantitative trait network. *Public Library of Science Genetics*, 5, e1000587.

Klie M., Menz I., Linde M., and Debener T. (2013). Lack of structure in the gene pool of the highly polyploidy ornamental chrysanthemum. *Molecular Breeding*, 32: 339–348.

Koyama M. L., Levesley A., Koebner R. M. D., Flowers T. J., and Yeo A. R. (2001). Quantitative trait loci for component physiological traits determining salt tolerance in rice. *Plant Physiology*, 125: 406–422.

Liu M., Zhang S., Liang H., and Zhen Z. (2008). AFLP analysis on the genetic diversity of some chrysanthemum species. *Journal of Agricultural University of Hebei*, 31: 48–59.

Mannai Y. E., Shehzad T., and Okuno K. (2011). Variation in flowering time in sorghum core collection and mapping of QTLs controlling flowering time by association analysis. *Genetic Resources and Crop Evolution*, 58: 983–989.

Martin C., Uberhuaga E., and Perez C. (2002). Application of RAPD markers in the characterisation of chrysanthemum varieties and assessment of somaclonal variation. *Euphytica*, 127: 247–253.

Mazzucato A., Papa R., Bitocchi E., Mosconi P., Nanni L., Negri V., Picarella M.E., Siligato F., Soressi G.P., Tiranti B., and Veronesi F. (2008). Genetic diversity, structure and marker-trait associations in a collection of Italian tomato (*Solanum lycopersicum* L.) landraces. *Theoretical and Applied Genetics*, 116: 657–669.

Misra S., Mandal T., and Vanlalruati Das S. K. (2013). Correlation and path coefficient analysis for yield contributing parameters in spray chrysanthemum. *Journal of Horticulture Letters*, 3: 14–16.

Nakano Y., Higuchi Y., Sumitomo K., and Hisamatsu T. (2013). Flowering retardation by high temperature in chrysanthemums: involvement of *FLOWERING LOCUS T-like 3* gene repression. *Journal of Experimental Botany*, 64: 909–920.

Nishi N., Muta T., Ito Y., Nakamura M., and Tsukiboshi T. (2009). Ray speck of chrysanthemum caused by *Stemphylium lycopersici* in Japan. *Journal of General Plant Pathology*, 75: 80–82.

Nordborg M., and Tavare S. (2002). Linkage disequilibrium: what history has to tell us? *Trends in Genetics*, 18: 83–90.

Portis E., Mauro R. P., Barchi L., Acquadro A., Mauromicale G., and Lanteri S. (2014). Mapping yield-associated QTL in globe artichoke. *Molecular Breeding*, 34: 615–630.

Pritchard J., Stephens M., and Donnelly P. (2000). Inference of population structure using multi locus genotype data. *Genetics*, 155: 945–959.

Rakshit A., Rakshit S., Singh J., Chopra S. K., Balyan H. S., Gupta P. K., and Bhat S. R. (2010). Association of AFLP and SSR markers with agronomic and fiber quality traits in *Gossypium hirsutum* L. *Journal of Genetics*, 89: 155–162.

Roein Z., Hassanpour Asil M., Sabouri A., and Dadras A. R. (2014). Genetic structure of chrysanthemum genotypes from Iran assessed by AFLP markers and phenotypic traits. *Plant Systematics and Evolution*, 300: 493–503.

Rostoks N., Ramsay L., MacKenzie K., Cardle L., Bhat P. R., Roose M. L., Svensson J. T., Stein N., Varshney R. K., Marshall D. F., Graner A., Close T. J., and Waugh R. (2006). Recent history of artificial outcrossing facilitates whole-genome association mapping in elite inbred crop varieties. *Proceedings of the National Academy of Sciences*, 103: 18656–18661.

Saghai-Maroof M. A., Soliman K. M., Jorgensen R. A., and Allard R. W. (1984). Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences*, 81: 8014–8018.

Saïdou A. A., Clotault J., Couderc M., Mariac C., Devos K. M., Thuillet A. C., Amoukou I. A., and Vigouroux Y. (2014). Association mapping, patterns of linkage disequilibrium and selection in the vicinity of the PHYTOCHROME C gene in pearl millet. *Theoretical and Applied Genetics*, 127: 19–32.

Shao Q. S., Guo Q. S., Deng Y. M., and Guo H. P. (2010). A comparative analysis of genetic diversity in medicinal *Chrysanthemum morifolium* based on morphology, ISSR and SRAP markers. *Biochemical Systematics and Ecology*, 38: 1160–1169.

Sun C. Q., Chen F. D., Teng N. J., Liu Z. L., Fang W. M., and Hou X. L. (2010). Interspecific hybrids between *Chrysanthemum grandiflorum* (Ramat.) Kitamura and *C. indicum* (L.) Des Moul and their drought tolerance evaluation. *Euphytica*, 174: 51–60.

Teixeira da Silva J. A. (2004). Ornamental chrysanthemums: improvement by biotechnology. *Plant Cell, Tissue and Organ Culture*, 79: 1–18.

Vos P., Hogers R., Bleeker M., Reijans M., Delee T. V., Hornes M., Friters A., Pot J., Paleman J., Kuiper M., and Zabeau M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, 23: 4407–4414.

Wang X. G., Wang H. B., Chen F. D., Jiang J. F., Fang W. M., Liao Y., and Teng N. J. (2014). Factors affecting quantity of pollen dispersal of spray cut chrysanthemum (*Chrysanthemum morifolium*). *BMC Plant Biology*, 14: 5.

Yagi M., Yamamoto T., Isobe S., Tabata S., Hirakawa H., Yamaguchi H., Tanase K., and Onozaki T. (2014). Identification of tightly linked SSR markers for flower type in carnation (*Dianthus caryophyllus* L.). *Euphytica*, 198: 175–183.

Yan W. G., Li Y., Agrama H. A., Luo D., Gao F., Lu X., and Ren G. (2009). Association mapping of stigma and spikelet characteristics in rice (*Oryza sativa* L.). *Molecular Breeding*, 24: 277–292.

Yu J., and Buckler E. S. (2006). Genetic association mapping and genome organization of maize. *Current Opinion in Biotechnology*, 17: 155–160.

Yu J., Pressoir G., Briggs W. H., Bi I. V., Yamasaki M., Doebley J. F., McMullen M. D., Gaut B. S., Nielsen D. M., Holland J. B., Kresovich S., and Buckler E. S. (2006). A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. *Nature Genetics*, 38: 203–208.

Yu S., Zhang F., Wang X., Zhao X., Zhang D., Yu Y., and Xu J. (2010). Genetic diversity and marker-trait associations in a collection of Pak-choi (*Brassica rapa* L. ssp. chinensis Makino) accessions. *Genes Genome*, 32: 419–428.

Zhang F., Chen S., Chen F., Fang W., Deng Y., Chang Q., and Liu P. (2011). Genetic analysis and associated SRAP markers for flowering traits of chrysanthemum (*Chrysanthemum morifolium*). *Euphytica*, 177: 15–24.

Zhang F., Chen S., Chen F., Fang W., and Li F. (2010). A preliminary genetic linkage map of chrysanthemum (*Chrysanthemum morifolium*) cultivars using RAPD, ISSR and AFLP markers. *Scientia Horticulturae*, 125: 422–428.

Zhang F., Jiang J., Chen S., Chen F., and Fang W. (2012). Mapping single-locus and epistatic quantitative trait loci for plant architectural traits in chrysanthemum. *Molecular Breeding*, 30: 1027–1036.

Zhao J., Paulo M. J., Jamar D., Lou P., van Eeuwijk F.,

Bonnema G., Vreugdenhil D., and Koornneef M. (2007). Association mapping of leaf traits, flowering time, and phytate content in *Brassica rapa*. *Genome*, 50: 963–973.

Zhao W.G., Chung J. W., Kwon S. W., Lee J. H., Ma K. H., and Park Y. J. (2013). Association analysis of physicochemical traits on eating quality in rice (*Oryza sativa* L.). *Euphytica*, 191: 9–21.

SUPPLEMENTAL DATA

Supplemental Table 1. The chrysanthemum genotypes investigated in this study.

Code	Name	Breeder's reference	Code	Name	Breeder's reference
Chr1	Khorshid	BR421	Chr25	Nasrin	BR176
Chr2	Sharif	BR217	Chr26	Shafia	BR422
Chr3	Ashoob	BR154	Chr27	Elham	BR44
Chr4	Keshavarz	BR764	Chr28	Dorsa	BR207
Chr5	Sharareh	BR272	Chr29	Donya	BR338
Chr6	Iran	BR186	Chr30	Keivan	BR215
Chr7	Baran	BR499	Chr31	Paniz	BR81
Chr8	Bita	BR387	Chr32	Unknown2	Unknown
Chr9	Kia	BR41	Chr33	Ofogh	BR27
Chr10	mahboob	BR318	Chr34	Arman	BR100
Chr11	Mir	BR196	Chr35	Maria	BR209
Chr12	Takapo	BR126	Chr36	Aria	BR378
Chr13	Poloneh	BR765	Chr37	Unknown3	Unknown
Chr14	Kiana	BR286	Chr38	Nasiri	BR440
Chr15	Unknown1	Unknown	Chr39	Afsoon	BR278
Chr16	Afrooz	BR113	Chr40	Simin	BR26
Chr17	Toloa	BR9	Chr41	Azadi	BR117
Chr18	Azar	BR86	Chr42	Padideh	BR57
Chr19	Helia	BR524	Chr43	Mehr	BR408
Chr20	Pajohesh	BR500	Chr44	Kafi	BR262
Chr21	Parastoo	BR141	Chr45	Shafagh	BR506
Chr22	Kiarash	BR145	Chr46	Gita	BR159
Chr23	Parvaneh	BR542	Chr47	Kimia	BR49
Chr24	Azarakhsh	BR425	Chr48	Unknown4	Unknown

Supplemental Table 2. The sequence of adapters and primers used for the AFLP analysis.

Primer/adapter	Code	Sequence
*Eco*RI	*Eco*RI-B-F	5'-CTCGTAGACTGCGTACC-3'
	*Eco*RI-B-R	5'-CATCTGACGCATGGTTAA-3'
*Mse*I	*Mse*I -B-F	5'- GACGATGAGTCCTGAG-3'
	*Mse*I-B-R	5'-TACTCAGGACTCAT-3'
Pre-amplification primer		
*Eco*RI+0	*Eco*RI-A	5'-GTAGACTGCGTACCAATTC-3'
*Mse*I +0	*Mse*I-A	5'-GATGAGTCCTGAGTAA-3'
Selective primers		
*Mse*I +3		
*Mse*I+ CAC	M-CAC	5'-GATGAGTCCTGAGTAACAC-3'
*Mse*I+ CAG	M-CAG	5'-GATGAGTCCTGAGTAACAG-3'
*Mse*I+ CAA	M-CAA	5'-GATGAGTCCTGAGTAACAA-3'
*Mse*I+ CTT	M-CTT	5'-GATGAGTCCTGAGTAACTT-3'
*Mse*I+ CTG	M-CTG	5'-GATGAGTCCTGAGTAACTG-3'
*Eco*RI+3		
*Eco*RI+ ACA	E-ACA	5'-GTAGACTGCGTACCAATTCACA-3'
*Eco*RI+ AAC	E-AAC	5'-GTAGACTGCGTACCAATTCAAC-3'
*Eco*RI + AAG	E-AAG	5'-GTAGACTGCGTACCAATTCAAG-3'
*Eco*RI+ AGA	E-AGA	5'-GTAGACTGCGTACCAATTCAGA-3'
*Eco*RI+ ACC	E-ACC	5'-GTAGACTGCGTACCAATTCACC-3'
*Eco*RI+ ACG	E-ACG	5'-GTAGACTGCGTACCAATTCACG-3'
*Eco*RI+ AA	E-AA	5'-GTAGACTGCGTACCAATTCAA-3'