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ASSOCIATION MAPPING OF QUALITY TRAITS WITH SSR MARKERS IN TALL FESCUE (*FESTUCA ARUNDINACEA SCHREB*.)

Y. Lou[1+], X. Sun[1+], Y.Chao[2], E.Amombo[1], H.Wang[1], F.Song[1],Q.Xu[3*], and Y. Zhuge[1*]

[1]National Engineering Laboratory for Efficient Utilization of Soil and Fertilizer Resources, College of Resources and Environment, Shandong Agricultural University, Daizong Road, Tai'an City, Shandong, 271018, P. R. China
[2]AgriculturalAdministerOffice, Tai'an Hi-Tech Industrial Development Zone, Nantianmen Street, Tai'an City, Shandong, 271000, R. P. China; [3]College of Agronomy, Hunan Agricultural University, Nongda Road, Changsha City, Hunan, 410128, P.R.China
[*]Corresponding Author's Email:zhugeyp@sdau.edu.cn; 1075324207@qq.com

ABSTRACT

Identification of the association between molecular markers and quality traits would promote the efficient utilization, conservation and management of the tall fescue germplasm. Thus, 59 tall fescue accessions from different origins (16 cultivars; 19 from America; 15 from Europe; 2from Africa and 7 from Asia), were evaluated for quality traits and genetic diversity with 90 simple sequence repeat (SSR) markers. Significant variations were observed among 59 tall fescue accessions in dry matter (DM), crude protein (CP), crude fat (CF),and CP were negatively correlated withDM (r=0.269) and CF (r=0.254), respectively. Three sub-populations were identified within the collections but no obvious relative kinship (*K*) was found. The GLM model was used to describe the association between SSR markers and quality traits. 41 SSR markers associated with quality traits were observed. In addition, 26 markers were significantly associated with CP; 12 markers with CF and 4 markers with DW. Notably, M100 marker was significantly both associated with CF and DM. Moreover, six significant associations were observed between CP and M213, M214, M215, M217, M218, and M216 markers, and the explained ratio of genetic variation were exceed 20 %. These identified marker alleles associated with quality traits could provide important information and markers for molecular-assisted breeding in tall fescue.

Key words: Association mapping; SSR; Tall fescue; Quality traits.

INTRODUCTION

Tall fescue (*Festucaarundinacea* Scherb.) is an important perennial cool-season forage and turf grass belonging to the grass family poaceae, subfamily pooideae, and tribe poaceae that is distributed throughout the temperate regions of the globe (Hoveland 2005). Due to the character of widely adaption, natural populations are found in Europe, North-West Africa, North America, West and Central Asia (Niazkhani *et al*., 2014). Nowadays, tall fescue is used extensively as forage in pastoral industry. Therefore, elite cultivars with good forage quality are required in modern pastoral industries.

A better understanding of the genetic variability from the available germplasm collection is a key component in selecting rational parents for hybridization and breeding. However, poor documentation and mismanagement of genetic variability may lead to diversity loss and hence the utilization limiting. Generally, the wide variations in germplasm might be attributed to genotypic and environmental aspects (Dutta *et al*., 2013). Previously research indicated that severe genetic erosion in Tunisia could have reduced the substantial variation in tall fescue cultivars (Chtourou-Ghorbel *et al*., 2011). Therefore, it is necessary to detail the forage quality traits of tall fescue germplasm for the effective utilization. In order to provide a reasonable representation of the overall genetic performance of tall fescue germplasm, a large sample size is an essential prerequisite. However, describing the forage quality traits is still costly and time-consuming.

Several previous studies have utilized various molecular markers e.g. RFLPs, RAPD, AFLPs, and SSRs to assess genetic diversity, andmarker-assisted selection (MAS)for molecular breeding (Saha *et al*., 2006; Sun *et al*., 2011; Rostami *et al*., 2015). The SSR markers offer several advantages such as co-dominance, genome abundance, high polymorphisms, locus specificity, good reproducibility and random distribution throughout the genome (Sun *et al*., 2011). As a result, SSR markers have been widely applied in traits and marker association of plants, such as plant height and spike weight in tall fescue (Lou *et al*., 2015), fiber content in cotton (Abdurakhmonov *et al*., 2008), and kernel size and milling quality in wheat (*Triticumarstivum* L.) (Breseghello *et al*., 2006). The SSR markers in tall fescue were available in enriched genomic libraries (Saha *et al*., 2006), hence enhanced their utilization for cultivar identification and genetic diversity assessment in tall fescue.

MAS is a widely applied technology that facilitates crop selection and breeding. The initial requirement for implementation of MAS in breeding is to identify the quantitative traits loci for characters of interest and linked molecular markers (Collard *et al.*, 2005). Association mapping, also called linkage disequilibrium, or LD mapping, is a powerful technique in searching for genotype-phenotype correlation in unrelated individuals. As a promising approach for plant breeders, association mapping is usually faster and more effective, and can eliminate the main setbacks of classical linkage analysis such as prolonged time and expensive generation of specific genetic populations. Furthermore, this approach can assess larger number of alleles and increase mapping resolution (Yu and Buckler, 2006). Association mapping has been successfully applied in genetic mapping of plants (Thornsberry *et al.*, 2001). However, the application of association mapping in detecting links between markers with quality traits in tall fescue is still undocumented. Therefore, the objectives of present study were to estimate quality traits diversity among a core collection of 59 tall fescue accessions with different geographic regions; and to identify the association of SSR markers with quality traits. These resultswill contribute to the conservation, utilization of the tall fescue germplasm effectively.

MATERIALS AND METHODS

Plant materials and growth conditions. For quality traits and molecular genetic diversity analysis, fifty-nine accessions including sixteen commercial cultivars were employed in this research (supplementary table 1). All accessions were obtained from the United States Department of Agriculture. The research was conducted at germplasm conservation garden of Wuhan Botanical Garden, Chinese Academy of Science, Wuhan, China from 2012 to 2014. The climate in this region is of a northern subtropical type, with a warm, humid summer, and an obvious altitudinal change.

Initially, a single plump seed of each accession was allowed to germinate on a layer of filter paper (pre-soaked in distilled water) in Petri dishes in May 2012. All Petri dishes were placed in the dark at 20 ºC until germination, and then transferred to the growth container (LSC-339CF; Xingxing Group Co., Zhejiang, China) with 14 h photo-period, and light intensity of 300-500 μ mol photons m^{-2} s^{-1} natural sunlight. After 14 days, all accessions were transferred to plastic containers (15 cm tall and 14 cm diameter) filled with a mixture of cultivation medium and sand (1: 1, v/v). To maintain genetic uniformity, each accession was cloned multiple times by tillers. All the plastic containers were placed in a walk-in growth room with daily maximum and minimum temperature of 24 ºC and 20ºC, with a 14-h photo-period, and a light intensity of 300 μ mol photons m^{-2} s^{-1} at canopy height, respectively. During the establishment period, plants were watered daily to maintain well watered condition, and fertilized weekly with half-strength Hoagland's solution (Hoagland and Amon, 1950). The grasses were mowed every week at a height of 7 cm.

In Sep, 2012, the field experiment plots were fertilized with 49 kg N ha^{-1}, 98 kg P ha^{-1}, 98 kg K ha^{-1}. Peat soil with 2-cm deep was applied, and then the soil subjected to deep-tillage for 25 to 30cm, raked for smoothness, and then covered with 2 cm-depth sand. The 59 accessions in plastic containers were transplanted to the experimental field in a 1.5×1.5 m-lattice in October, 2012. Randomized block plots with three replicates were designed in this research. Compound fertilizer with the ratio of N: P: K at 21: 6: 13 and urea were applied alternatively with the amount of 49 kg N ha^{-1}, and fertilized 7 times in 2013. All plants were moved to the height of 10 cm in 13 June, 2013, and then collected samples, and no mowing was conducted at other time during the whole experimental period. In April, 2014, the tall fescue was fertilized with compound fertilizer followed by urea in May to provide 49 kg N ha^{-1}, respectively. Similarly, in 7 Jun 2014, the plants were mowed to the height of 10 cm, and followed by subsequent sample collection. Perimeter pop-up gear-driven sprinkler with heads positioned at 3.5 m were employed, and irrigation plus rainfall were also allowed to prevent the tall fescue accessions from wilting. After irrigation, water was applied to wet the entire root zone. The experiment fields were syringed occasionally during sunny and rain-free periods.

Quality traits measurement: For forage quality analysis at harvest, a second set of random samples of 100 g biomass from each plot was taken. Tissue samples were weighted and oven dried at 105 °C for 30 min, and then oven dried at 70 °C for 72 h to constant weight. Dried samples were ground through the 1-mm screen of a cyclone mill. Dry matter content was accessed according to method described by Zhang *et al.* (2002). Crude protein (CP) was quantified based on total nitrogen. Total nitrogen of dry samples was determined using an automatic micro-Kjeldahl method described by Yahaya *et al.* (2002). Crude fat was determined by the diethylether extraction method without hydrolysis using a Soxhlet extractor (Escribano *et al.*, 1997).

DNA isolation and SSR analysis: Total genomic DNA was extracted using the modified CTAB procedure as described by Xie *et al.* (2012). A set of 90 published genome-wide SSR markers (Saha *et al.*, 2005; 2006) mapped in 22 linkage groups in tall fescue were analyzed (supplementary table 2). PCR protocol as following: 1 cycle of 10 min at 95 ºC; followed by 25 cycles of 50 s at 95 ºC, 50 s at 68 ºC with a decrease of 0.6 ºC in each consequent cycle, 60 s at 72 ºC. Another 15 cycles was 50 s at 95 ºC, 50 s at 54 ºC, 60 s at 72 ºC. The reaction ended with a 10 min extension at 72 ºC. ABI 3730 DNA

Sequence (Applied Biosystems Inc., Foster City, CA, USA) was used in the PCR amplified fragments separation. Alleles were scored by GeneMarker 1.5 software (Soft Genetics, LLC, State College. PA, USA) and checked twice manually for accuracy. Presence (1) and absence (0) data were employed for SSR analysis.

Population Structure: The Bayesian model-based clustering method was carried out in STRUCTURE 2.3.1 software. The length of burn-in period and the number of Markov Chain Monte Carlo (MCMC) replications after burn-in were all assigned at 100,000 with an admixture and allele frequencies correlated model. The structure was run ten times by setting pre-defined K ranging from 1 to 15 using admixture models (Evanno *et al*., 2005). 15 independent runs were operated 100,000 interactions of each run after burn-in of 100,000 for a value of K setting from 1 to 5.

Associating mapping: Three models were used to access the effects of relative kinship (K) and population structure (Q) for marker-trait associations. The Q model was performed using general linear model (GLM) in TASSEL 2.1 software (Bradbury *et al*., 2007). The data of polymorphism SSR locus was response variable, while the quality traits were independent variables. The significant threshold for marker-trait association was set at P<0.01.

Statistical analysis: The experiment was arranged in a completely randomized block design with four replications. All quality traits data were averaged over two years. All data were performed with the SPSS statistical software package (version 20.0; SPSS, Chicago, IL, USA).

RESULTS

Genetic variation in quality traits of tall fescue accessions: Frequency distributions of each quality trait (DM, CP, and CF) among all accessions exhibited a normal distribution as shown in Figure 1. The CF regime (20.56%) exhibited a greater coefficient of variation than other traits (Table 1). In contrast, DM had the lowest CV (11.54 %). Among all the accessions, DM ranged from 26.38 % to 43.71 %, CP ranged from 4.28 % to 9.46 %, and CF ranged from 2.08 % to 6.30 %. Correlation analysis showed that CP was significantly correlated to DM (r=0.269) and CF (r=0.254), respectively (Table 2).

No significant difference was observed in DM and CF between wild accessions and commercial cultivars. The CP in African and Asian groups were lower than commercial cultivars (Table 3). Significant variation in CV of quality traits was also observed among accessions from different geographic regions (Table 4). Commercial cultivars had the lowest CV in DM (7.98 %), while Europe group had the highest CV (14.09 %). African group had the highest CV in CP (24.08 %), while European group had the lowest CV (12.75 %). Asian group had the highest CV in CF (29.83 %), while African group had the lowest CV (12.83 %).

Genotype and Population structure analysis: A total of 1010 SSR alleles were obtained from the 90 SSR markers across the 59 tall fescue accessions with an average of 11.22 alleles per locus. The allele numbers of SSR marker varied from 3 to 27 alleles per marker, and all of the individuals were successfully distinguished by these bands as shown in Figure 2. According to STRUCTURE analysis results based on Bayesian clustering approach model, a significant population structure was detected among the 59 gene bank accessions. Three structure groups (GI, GII and GIII) were identified in the collection of 59 tall fescue accessions. GI contained thirty-five accessions, covering all the geographic regions (thirteen commercial cultivars, nine accessions from Africa, nine accessions from Europe, and three accessions from Asia). GII contained sixteen accessions, mainly wild materials from Africa, Europe, and Asia. GIII contained only eight wild accessions.

Association analysis: A total of forty-one associations were identified between the SSR markers and the three quality traits (P< 0.05) (Table 5). With regard to quality traits, twenty-six markers were significantly associated with CP (with the percentage of the total variation explained by each marker ranged from 7.6 % to 25.4 %), twelve associated markers were associated with CF (with the percentage of the total variation explained by each marker ranged from 6.7 % to 13.0 %), four markers were associated with DW (with the percentage of the total variation explained by each marker ranged from 8.2 % to 14.9 %). Notably, M100 was associated with both CF and DM, with the percentage of total variation explanation was 12.8 % and 12.7 %, respectively.

Table 1 Range of Dry matter (DM), Crude protein (CP), and Crude fat (CF) for 59 tall fescue accessions.

Traits	Maximum	Minimum	Mean	Std	CV
DM (%)	43.71	26.38	32.92	3.80	11.54
CP (%)	9.46	4.28	6.39	1.09	17.01
CF (%)	6.30	2.08	4.52	0.93	20.56

[a]Std. standard deviation; [b]CV coefficient of variation

Table 2. Pearson correlation coefficients among DM, CP, and CF for 59 tall fescue accessions.

Quality traits	DM	CP	CF
DM	1		
CP	0.269*	1	
CF	-0.095	0.254*	1

[a]*, Significant at P = 0.05 (2-tailed).

Table 3. The variation of quality traits in tall fescue accessions from diverse regions.

Quality traits	Origin				
	Cultivar	America	Europe	Africa	Asia
DM (%)	32.95±2.63ab	31.34±2.93b	33.73±4.74ab	30.40±3.54b	36.14±4.25a
CP (%)	7.01±1.07a	6.32±1.15ab	6.28±0.80ab	5.55±1.34b	5.50±0.79b
CF (%)	4.51±1.00a	4.49±0.88a	4.81±0.80a	4.63±0.59a	3.85±1.15a

[a]Notes: Values in a row followed by the same letter indicate no significant difference at *P*<0.5.

Table 4. Variation coefficient of quality traits in tall fescue accessions from diverse origins.

Quality traits	Cultivar	America	Europe	Africa	Asia
	(n=16)	(n=19)	(n=15)	(n=2)	(n=7)
DM (%)	7.98	9.34	14.09	11.63	11.77
CP (%)	15.26	18.16	12.75	24.08	14.37
CF (%)	22.27	19.61	16.70	12.83	29.83

Table 5. Association of SSR markers with percentage of reduction of quality traits of tall fescue accessions by corrected *P* values ($P<3.5\times10^{-4}$)

Trait	Marker	Marker_F	Marker_P	MarkerR2	Trait	Marker	Marker_F	Marker_P	MarkerR2
CP	M214	9.920016	0.000224	0.254267	CP	M131	3.376189	0.041824	0.105814
CP	M215	9.301232	0.000352	0.242585	CP	M246	3.353943	0.042656	0.105197
CP	M217	8.906063	0.000472	0.234908	CP	M127	3.309476	0.044371	0.10396
CP	M218	8.892811	0.000477	0.234648	CP	M125	3.275371	0.045735	0.103008
CP	M216	8.822077	0.000502	0.233254	CP	M121	3.23162	0.047548	0.101784
CP	M213	8.77674	0.000520	0.232358	CF	M253	3.822984	0.028377	0.120741
CP	M237	6.244105	0.003712	0.178293	CF	M165	4.551523	0.037628	0.074537
CP	M110	7.626618	0.007886	0.11582	CF	M32	3.836625	0.028042	0.121115
CP	M129	5.1493	0.009113	0.152199	CF	M183	4.394588	0.040937	0.072167
CP	M120	4.879398	0.011427	0.145482	CF	M106	4.360773	0.04169	0.071655
CP	M71	4.632822	0.014075	0.139243	CF	M48	3.320298	0.044103	0.106693
CP	M229	4.347734	0.017948	0.131902	CF	M11	4.20189	0.045437	0.069239
CP	M255	3.694572	0.031601	0.114551	CF	M12	4.041306	0.049599	0.066784
CP	M68	3.86944	0.027127	0.119271	CF	M169	8.48794	0.005259	0.129955
CP	M40	4.79	0.033053	0.076313	CF	M168	7.472837	0.008541	0.116366
CP	M252	3.63179	0.033389	0.112843	CF	M100	4.075567	0.022802	0.127619
CP	M243	3.61904	0.033765	0.112496	CF	M29	3.9387	0.025665	0.123906
CP	M235	3.572287	0.03518	0.111218	DM	M76	3.44419	0.03924	0.112605
CP	M122	3.488514	0.037873	0.108918	DM	M100	3.938329	0.025431	0.12667
CP	M132	3.488293	0.037881	0.108912	DM	M49	4.743284	0.012734	0.148629
CP	M239	3.47356	0.038376	0.108506	DM	M39	4.908069	0.030969	0.081568

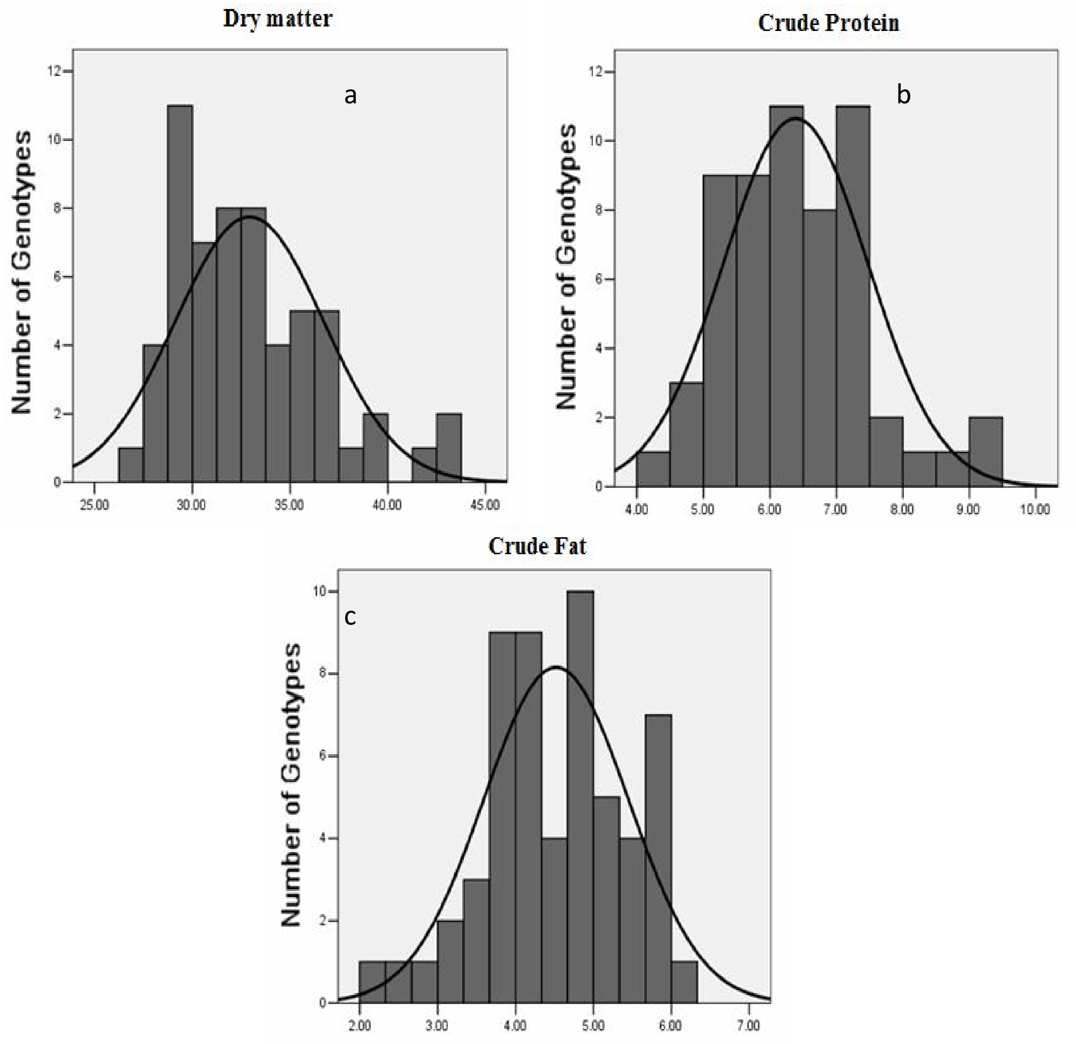

Fig. 1 Frequency distribution in morphological traits of tall fescue accessions

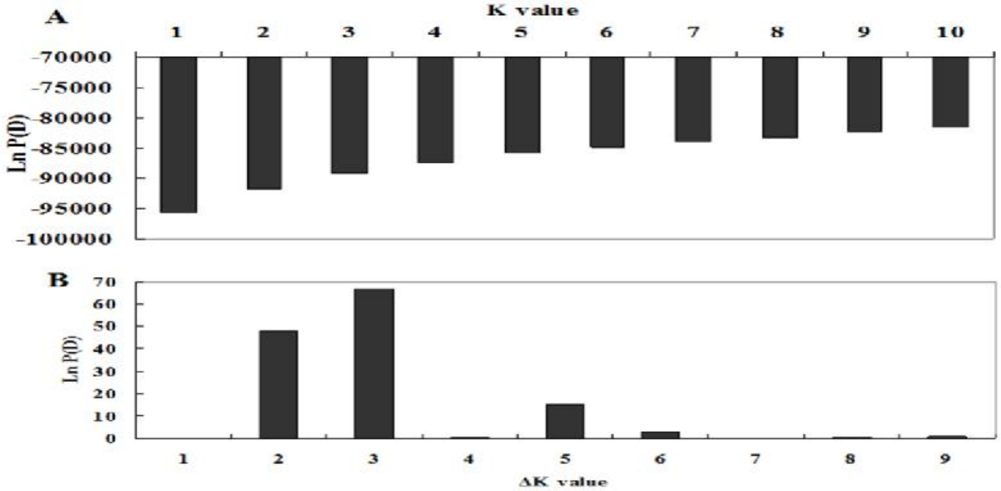

Fig 2 Calculation of true K of tall fescue accessions and (A) Evolution of the average logarithm probability of the data likelihoods (LnP(D)) for tall fescue genotypes; (B) Magnitude of Δk for each K value according to Evanno *et al. (2005).*

DISCUSSION

Quality traits diversity: Plant germplasm resources, comprised of wild accession, commercial cultivars, and their wild relatives, are important reservoirs of natural genetic variations. A better understanding of the genetic variation across species is crucial for their effective conservation and utilization. Therefore, knowledge regarding genotypic diversity is critical for improving of crop plants productivity. Meanwhile, this information has been successfully used for efficient germplasm management and genotype selection for various breeding purposes (Matus *et al*., 2002). Many association analysis studies have been done on crops such as wheat (Baenziger *et al*., 2001), cotton (Abdurakhmonov *et al.*, 2008), and alfalfa (Julier*et al*., 2000). Several studies have revealed that high diversity in quality traits could be a useful tool for germplasm collection. In this research, significant genetic variation was observed in quality traits among 59 tall fescue accessions. These results were consistent with the observations in Cameroonian yam *Saccharumspontaneum* (Liu *et al*., 2014).

Cultivar variation is an important indicator for crop breeders to evaluate the genetic range of germplasm. The CF regime exhibited higher diversity with the greatest CV (20.56 %), which suggested good potential for selection in a breeding program. Furthermore, one quality trait could be used to predict another trait based on their correlation. Correlation analysis showed that CP was significantly correlated to DM (r=0.269) and CF (r=0.254), respectively. This knowledge regarding the relationship between the observed quality traits is critical for the effective designing of improved breeding strategies, and eventually could facilitate tall fescue breeding. On the contrary, the least variability observed in DM revealed their low efficiency to evaluate genetic variability in tall fescue. With regard to geographic origin, no significant difference was detected between wild group and commercial cultivar group, and this is not similar to morphological traits (Lou *et al*., 2015).

Association mapping: Currently, great attention has been shifted towards association mapping to a wide range of organisms, e. g., maize (Thornsberry*et al*., 2001). The information on the location of the genome regions controlling traits of interest was provided by the significant MAS. Jin *et al*. (2010)identified association mapping between quality traits and SSR markers in rice (*Oryza sativa* L.), and reported that five SSRs markers were associated with amylase content, while seven SSRs markers were associated with pasting temperature. Association mapping was also applied on wheat germplasm; a total of 14 SSRs loci were significantly associated with agronomic traits, and the percentage of the total variation explained by each marker was from1.8 % to 16.3 % (Yao *et al*., 2009).

Previous studies indicated that the key step to association analysis is to separate the population structure from the genetic linkage. Samples with diverse geographic origins employed in association analysis might contain familial relatedness and population structure (Yu and Buckler, 2006). Without being correctly controlled, the existence of genetic structure in the populations could lead to false-positive results (Yu *et al*., 2011). Therefore, population structure estimation was the prerequisite for association analysis to reduce this risk. With regard to the present research, tall fescue populations contained population structure but no obvious familial relationships were detected. Forty-one associations were identified between the SSR markers and the three quality traits ($P< 0.05$). These markers were confirmed to be efficient in correlating quality traits that have not been reported. Alternatively, the markers were confirmed to be posses high potential of correlating with quality traits which require further investigation. Notably, only one marker, M100 was associated with both CF and DM, with the percentage of total variation explanation of 12.8 % and 12.7 %, respectively. Six significant associations between CP and M213, M214, M215, M217, M218, and M216 markers, and the explained ratio of genetic variation was exceed 20 %, and these markers may be effectively applied in future tall fescue breeding. Meanwhile, quantitative trait loci (QTL) associated with forage digestibility and biomass yield were also identified based on the microsatellite map of tall fescue (Saha*et al*., 2009). However, whether the identified markers are located within the known QTL interval need to be further revealed.

In summary, large variations in DM, CP, and CF were observed among 59 accessions of tall fescue. The CP regime had negative correlation with DM (r=0.269) and CF (r=0.254), respectively. Three sub-populations were observed without obvious relative kinship (*K*). The GLM model was recognized as the best model to describe association between SSR and quality traits. Fifty-one SSR markers were detected that associated significantly with quality traits. In particular, M100 was associated with both DM and CF. Notably; the explained ratios of genetic variation of CP by six markers (M213, M214, M215, M217, M218, and M216) were exceed 20 %. These markers would enhance efficiency in desirable allele selection for elite cultivars in tall fescue breeding in future.

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