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Identification of genetic loci associated with crude protein and mineral concentrations in alfalfa (*Medicago sativa*) using association mapping

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Abstract

Background: Alfalfa (*Medicago sativa*) is one of the most important legume forage species in China and many other countries of the world. It provides a quality source of proteins and minerals to animals. Genetic underpinnings for these important traits, however, are elusive. An alfalfa (*M. sativa*) association mapping study for six traits, namely crude protein (CP), rumen undegraded protein (RUP), and four mineral elements (Ca, K, Mg and P), was conducted in three consecutive years using a large collection encompassing 336 genotypes genotyped with 85 simple sequence repeat (SSR) markers.

Results: All the traits were significantly influenced by genotype, environment, and genotype × environment interaction. Eight-five significant associations ($P < 0.005$) were identified. Among these, five associations with Ca were repeatedly observed and six co-localized associations were identified.

Conclusions: The identified marker alleles significantly associated with the traits provided important information for understanding genetic controls of alfalfa quality. The markers could be used in assisting selection for the individual traits in breeding populations for developing new alfalfa cultivars.

Keywords: Alfalfa, Association mapping, Crude protein, Mineral elements, Simple sequence repeat (SSR)

Background

Alfalfa (*Medicago sativa*) has long been cultivated as the most important legume forage crop in the world. The widespread use of alfalfa is due to its high forage quality. Alfalfa forage is high in crude protein (CP), but its rumen undegraded protein (RUP) content is low. Most proteins of alfalfa are degraded in rumen into small N-containing molecules, instead of essential amino acids that are required by animals, subsequently decreasing the utilization value of alfalfa N and consequently releasing to the environment and causing pollutions [1, 2]. K, Ca, Mg, and P are essential minerals to animals, which are involved in various physiological activities regulating

metabolisms [3], affecting growth and development, and improving the quality of animal products. For example, an increased concentration of K in diet could reduce trans-fatty acids in milk [4], prevent metabolic alkalosis and decrease the risk of hypocalcemia and milk fever in cows [5]. Thus, identification of genes that control aforementioned forage quality component traits will provide insights for alfalfa breeding programs.

Most quality traits, like CP, RUP, and mineral elements, are quantitative traits coded by multiple genes, influenced by environment, and complexed by genotype by environment interaction. Traditional linkage mapping is the most common approach to detect quantitative trait loci (QTLs) conditioning complex traits in plants. To date, several QTL studies in mineral elements have been reported based on linkage mapping investigations in *Oryza sativa* [6, 7], *Triticum aestivum* [8, 9], and *Brassica napus* [10],

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Jia *et al. BMC Plant Biology* (2017) 17:97

Page 2 of 7

and *Zea mays* [11, 12]. Compared with linkage analysis, association mapping is a recently-emerged alternative robust tool to overcome the restriction of classical QTL mapping [13]. It has been widely applied in the major crop species to detect QTLs by establishing marker-trait associations [14–18]. Recently, Huang et al. [19], and Nawaz et al. [20], respectively reported marker-trait associations (MTAs) for mineral elements in rice using association mapping. But for alfalfa, little information is available for the genetic structures in CP, RUP and mineral elements, by linkage analysis or association mapping. In the present study, genome wide association (GWAS) approach was used to investigate SSR markers associated with the six quality traits in a core collection of alfalfa.

Results

Phenotypic variations in an alfalfa association mapping population

The six measured traits for 336 alfalfa genotypes are given in Table 1. The average values of crude protein (CP) and Ca were slightly increased from 2013 to 2015, while K and Mg were just the opposite (Table 1; Fig. 1). All the datasets showed normal or nearly normal distributions (Table 1). The ANOVA results indicated that all the traits were significantly influenced by genotype, environment and genotype × environment interaction ($P < 0.001$) (Table 1). For genotype, the most notable effect was detected in Ca (H = 0.79) and the smallest in RUP (0.56) (Table 1). The most significant environment and genotype × environment effects were both observed in rumen undegraded protein (RUP), while the slightest in Mg and K (Table 1). The results indicated sufficient genetic variability existed in the alfalfa collection that was appropriate for the association mapping research. Interestingly, Pearson's phenotypic correlation coefficients were highly significant between the traits ($P < 0.001$) (Table 2). Among 15 pairs of the six traits, eight pairs showed positive correlations while the remaining seven pairs appeared to be negatively correlated (Table 2).
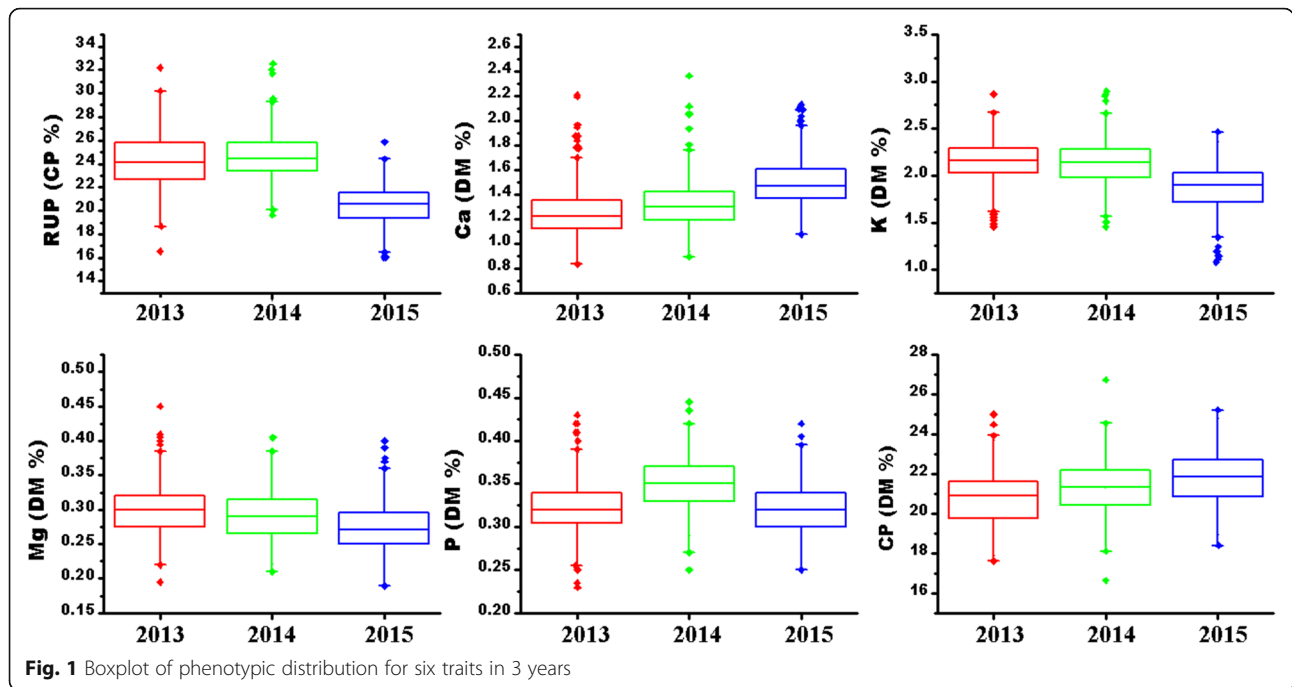
Association analysis

To control false positive trait-marker associations, three models, namely general linear model (GLM), Q, and Q + K, were compared with each other using the quantile-quantile (Q-Q) plot shown in Fig. 2. In general, the P value obtained from the Q + K model was more close to the expected P value than the other two models (Fig. 2). Using the respective mean phenotyping values of the 3 years, 256, 192, and 85 marker-trait associations (MTAs) were detected by GLM, Q, and Q + K model, respectively (Table 3). The result showed that the false positives were appropriately controlled by using the Q + K model. Therefore, the following analyses were based on the Q + K model.

Table 1 Phenotypic variation for six quality traits in alfalfa in 3 years

Traits	Year	Min	Max	Mean ± SD	Skewness	Kurtosis	H	Genotype	Year	Genotype ×year
CP (DM%)	2013	17.62	25.01	20.77 ± 1.33	−0.06	−0.13	0.67	10.14***	266.06***	3.29***
	2014	16.66	26.73	21.29 ± 1.40	−0.01	0.32				
	2015	18.39	25.21	21.85 ± 1.37	0.01	-0.45				
RUP (CP%)	2013	16.56	32.18	24.28 ± 2.35	0.06	0.11	0.56	8.51***	2176.46***	3.49***
	2014	19.65	32.52	24.63 ± 1.87	0.50	1.57				
	2015	16.05	25.88	20.54 ± 1.60	−0.01	0.10				
Ca (DM%)	2013	0.84	2.21	1.26 ± 0.20	1.36	3.62	0.79	13.79***	916.8***	2.87***
	2014	0.90	2.37	1.32 ± 0.90	1.13	3.79				
	2015	1.08	2.14	1.51 ± 0.19	0.82	0.74				
K (DM%)	2013	1.45	2.87	2.15 ± 0.23	−0.20	0.45	0.74	6.18***	436.11***	1.6***
	2014	1.46	2.90	2.14 ± 0.25	0.09	0.24				
	2015	1.08	2.47	1.88 ± 0.24	−0.45	0.49				
Mg (DM%)	2013	0.20	0.45	0.30 ± 0.04	0.60	0.88	0.76	10.15***	257.46***	2.53***
	2014	0.21	0.41	0.29 ± 0.03	0.33	0.27				
	2015	0.19	0.40	0.27 ± 0.04	0.68	0.86				
P (DM%)	2013	0.23	0.43	0.32 ± 0.03	0.35	1.20	0.59	5.82***	401.95***	2.41***
	2014	0.25	0.45	0.35 ± 0.03	0.10	0.20				
	2015	0.25	0.42	0.32 ± 0.03	0.32	0.17				

H, Broad-sense heritability
***indicate Significance at $P < 0.001$ levels

Jia *et al. BMC Plant Biology* (2017) 17:97

Page 3 of 7

Fig. 1 Boxplot of phenotypic distribution for six traits in 3 years

Based on the Q + K model, a total of 85 MTAs, respectively, 30 in 2013, 17 in 2014 and 38 in 2015, were detected significantly for the six measured traits with 46 SSR markers in at least 1 year (Additional file 1). For CP, three, two and eight MTAs were significant in 3 years, respectively, with the explained phenotypic variance (R^2) ranging from 2.88 to 3.81% (Additional file 1). For RUP, six, four, and two MTAs were identified to be significant in 3 years, respectively, with the R^2 from 2.69 to 4.80%. For Ca, eight, six, and ten significant MTAs were detected in 3 years, respectively, with the R^2 from 2.44 to 8.20%. For K, four, zero, and two MTAs were significant in 3 years, respectively, with the R^2 from 2.59 to 3.86%. For Mg, three, zero, and nine MTAs were detected in 3 years, respectively, with the R^2 from 2.58 to 5.27%. For P, six, five, and seven MTAs were detected in 3 years, respectively, with the R^2 from 2.43 to 3.69%.

Among the 85 MTAs, most were detected only in 1 year for CP, RUP, K, Mg, and P except those for Ca. Five MTAs with Ca were repeatedly observed in 2 years.

Table 2 Correlation analysis for six forage quality related traits in alfalfa

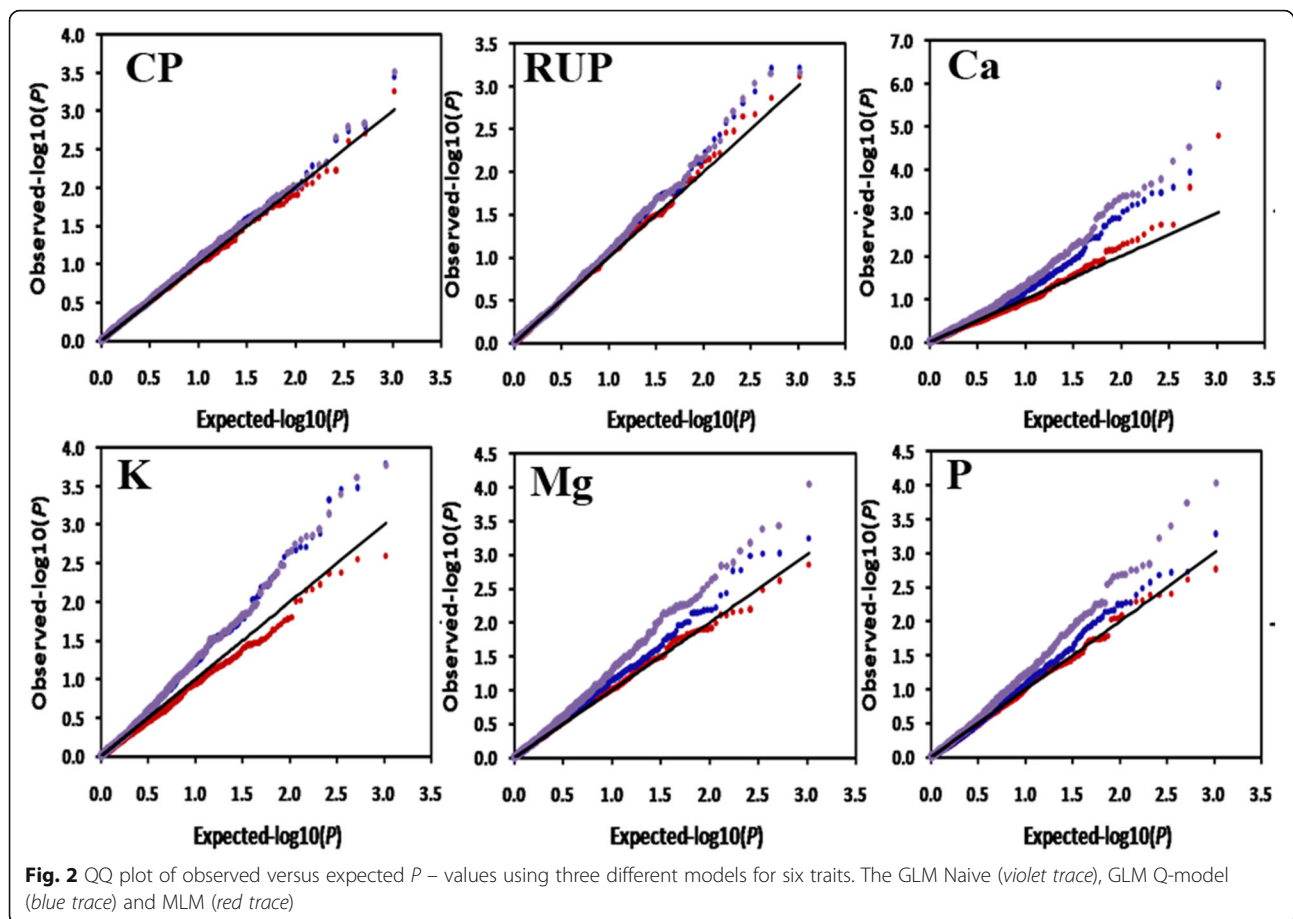|	CP	Ca	K	Mg	P
Ca	0.178[***]				
K	0.163[***]	−0.622[***]			
Mg	0.197[***]	0.704[***]	−0.525[***]		
P	0.497[***]	−0.399[***]	0.595[***]	-0.234[***]	
RUP	-0.368[***]	−0.558[***]	0.376[***]	−0.285[***]	0.212[***]

*** indicate Significance at $P < 0.001$

The alleles, m215_182 and m583_128, which located in chromosome one, were respectively detected in 2013 and 2015, and 2013 and 2014. The alleles, m13_170 and m13_173, which located in chromosome two, were detected in 2013 and 2015, 2014 and 2015, respectively. The allele m429_245 located in chromosome four was detected in 2014 and 2015 (Additional file 1).

Among these associated alleles, some alleles were found associated with multiple traits especially in 2015. Five alleles, m13_170, m19_128, m350_342, m429_245 and m53_151, co-associated with Ca and Mg in 2015, and the other three alleles m19_162, m359_175 and m46_123 co-associated with CP and P in 2015 (Additional file 1). One allele, m630_301 was co-associated with K and Mg in 2013, and m225_203 co-associated with Ca and P in 2014. The significant associations of the same alleles with multiple traits might be the result of pleiotropy.

Mining of elite alleles

The phenotypic effect of each allele that significantly associated with each of the six traits was shown in Additional file 1. In this study, the alleles with positive effects are considered to be elite alleles for all the six measured traits. Among the 13 alleles associated with CP, 11 showed positive effects. The allele m257_203 had the most positive phenotypic effect (3.16) in 2015. For RUP, five alleles had positive effects and allele m83_155 had the most positive phenotypic effect (3.59) in 2013. Only one allele m520_148 showed a positive effect (0.51) associated with K. The allele m630_301 had the most positive phenotypic effect (0.09) in 2013 among the 12

Jia *et al. BMC Plant Biology* (2017) 17:97

Page 4 of 7

Fig. 2 QQ plot of observed versus expected P – values using three different models for six traits. The GLM Naive (*violet trace*), GLM Q-model (*blue trace*) and MLM (*red trace*)

alleles associated with Mg. The allele m225_203 had the most positive phenotypic effect (0.09) associated with P in 2004. However almost all of elite alleles mentioned above were only associated in 1 year. The m13_170 was only one that stably associated with Ca over different years although it has small positive effect (0.06).

Discussion

In this study, alfalfa genotypes showed significant levels of genetic diversity ($P < 0.001$), as revealed by ANOVA of all the six traits, and the most traits were highly heritable, showing a broad variation among the alfalfa genotypes (Table 1). All the traits were significantly influenced by environment and genotype × environment interactions. Two important environment factors, precipitation and temperature, were investigated during the 3 years (Additional file 2). There was no any significant difference on the temperature during the 3 years. Whereas, some significant difference was observed for precipitation especially in April and May among 3 years which was the important development stage of the alfalfa. Obviously, the precipitation is the main factor leading to environmental variance and ultimately affect the six quality traits in the study. Correlation analysis of

different traits is considered very useful in exploring interrelationships. In the present study, numerous significant correlations were observed between the six different traits (Table 1). These correlations may be due to the impact of a single gene on multiple traits or co-association of physically closely located genes [20].

Association mapping is a reliable method for quickly identifying the loci responsible for natural variants in a target phenotype [21]. Recently, association mapping was also used to identify loci associated with the biomass yield and stem composition [22], and verticillium wilt resistance [23] in tetraploid alfalfa and forage yield and nutritive value in diploid alfalfa [24]. However, less information is known about the association of SSR loci with crude protein, RUP, and mineral elements in plant species. Huang et al. [19] reported a total of 20 marker-trait associations identified for the five mineral elements in rice. Nawaz et al. [20] identified 60 marker loci associated with eight grain elemental concentrations in brown rice. The present study is the first attempt in characterizing the alfalfa genotypes using genomic SSR markers for crude protein, RUP, and four mineral elements in a diverse set of worldwide collection of alfalfa accessions. A total of 85 MTAs was identified as associated with

Jia *et al. BMC Plant Biology* (2017) 17:97

Page 5 of 7

Table 3 Association summary for six measured traits in alfalfa using three models in 3 years

Traits	Year	GLM		Q		Q + K	
		No of alleles	R^2 (%)	No of alleles	R^2 (%)	No of alleles	R^2 (%)
CP	2013	5	2.13–4.09	5	2.13–4.01	3	2.89–3.81
	2014	3	2.35–4.02	2	2.21–3.75	2	3.25–3.58
	2015	8	2.10–3.87	10	2.31–3.84	8	2.88–3.54
RUP	2013	8	2.15–3.51	8	2.11–3.59	6	2.69–3.69
	2014	5	2.42–4.10	4	2.16–4.47	4	2.88–4.80
	2015	3	2.13–3.32	3	2.20–3.23	2	2.83–3.03
Ca	2013	29	2.06–7.40	24	2.26–6.92	8	2.47–5.99
	2014	20	2.16–9.65	13	2.09–8.95	6	3.15–8.20
	2015	30	2.13–6.10	19	1.94–6.67	10	2.44–6.95
K	2013	15	2.31–4.76	16	2.08–4.80	4	3.54–3.86
	2014	11	2.19–3.60	11	2.15–3.80	0	
	2015	17	2.16–7.09	14	2.15–5.03	2	2.59–3.79
Mg	2013	15	2.09–5.18	8	1.94–3.42	3	2.65–3.13
	2014	11	2.19–3.78	6	2.16–3.31	0	
	2015	26	2.24–6.46	18	2.04–5.23	9	2.58–5.27
P	2013	14	2.20–4.76	7	2.24–3.49	6	2.43–2.98
	2014	13	2.15–4.50	12	2.05–5.42	5	3.00–3.59
	2015	23	2.16–7.41	12	2.12–5.97	7	2.64–3.69

R^2, the explained phenotypic variance

measured traits based on the association analysis using the MLM model (Additional file 1). Most of the loci associated with the six traits were identified only in a specific year, suggesting their expression for these traits is significantly influenced by the environment. However, stable associations with Ca were identified in our study, such as the alleles m215_182 and m583_128 located in chromosome one, m13_170 and m13_173 located in chromosome two, m429_245 in chromosome four (Additional file 1). Therefore, these associated markers and identified genotypes with favorable alleles could be used for marker assisted selection in alfalfa breeding after validation.

It is reported that co-localized or pleiotropic associations may be helpful to reveal some important genomic regions or genes for the desirable traits [19]. In this study, several co-localized associations were detected. For example, m13_170 was found associated with Ca, and Mg in Chromosome two, suggesting that it may be possible to select for high Ca, and Mg lines using molecular markers in these regions (Additional file 1). m19_162 was found associated with crude protein and P in Chromosome two, indicating that it may be possible to select for high crude protein and P lines (Additional file 1). Similar results were reported in rice [19], *Aegilops tauschii* [25], and *Festuca arundinacea* [26]. Furthermore, the markers associated with more than one trait may be effectively used of improving more than one trait

in marker assisted selection. The phenomenon of co-localization may be caused by pleiotropy of the same gene involved in the metabolism and physiological processes of several elements or is the presence of clustered genes that are tightly linked responsible for the accumulation of different elements in rice grain [27].

Because of the complex nature of the studied traits and limited markers used, most of the experimental results showed poor repeatability and lower explained phenotypic variance (<10%) as indicated by the similar research [14, 18, 25, 26] . But some of elite alleles were still detected associated with the studies traits. These will be useful for molecular marker assist selection breeding in alfalfa compared to traditional phenotype-based selection.

Conclusions
The present study is the first attempt in characterizing the alfalfa genotypes using genomic SSR markers for CP, RUP, and four mineral elements (Ca, K, Mg and P) traits in a diverse set of worldwide collection of alfalfa accessions. Our results showed that this alfalfa association panel could be appropriate for association analyses targeting complex agronomics traits with optimal association models. The markers associated to the QTLs in the study could be effectively used in improving locally well adapted germplasm by marker assisted introgression of desirable alleles.

Jia *et al. BMC Plant Biology* (2017) 17:97

Page 6 of 7

Methods

Plant materials and experimental design

The association panel was consisted of a total of 336 individual genotypes from 75 cultivated tetraploid alfalfa accessions (Additional file 3) [28]. Nine Chinese accessions were obtained from the National Herbage Germplasm Bank of China; two accessions from Syria, one from Libya and one from Sudan provided by the Institute of Animal Science, Chinese Academy of Agricultural Science (Beijing, China).The other 62 accessions were provided by the USDA National Plant Germplasm System (NPGS). The field experiments were performed on the experimental station of the Institute of Dry Farming, Hebei Academy of Agriculture and Forestry Sciences in Hengshui, Hebei province, established in May 2012 (37°44′N; 115°42′E). The mean annual precipitation of 484 mm (65% in July and August), evaporation of 1670 mm, and average temperature of 13.2 °C, sunshine duration of 2546 h, relative humidity of 63%, frost-free period of 206 days in long-term. Soil type in this area is typically silty loam, with PH of 7.88, salt of 0.053%, organic matter of 16.5 g kg^{-1}, alkali–hydrolysable N of 60.4 mg kg^{-1}, available P of 12.5 mg kg^{-1}, available K of 201.7 mg kg^{-1} within the top 20 cm soil. The experimental design was reported previously [28].

Phenotyping

The analyses for the contents of crude protein (CP), rumen un-degraded protein (RUP), and mineral elements (Ca, K, Mg and P) were performed on all plant samples for 3 years (2013, 2014 and 2015). The biomass above the ground was harvested at the early flowering stage and dried at 60 °C for 48 h. Then plants were ground to pass a 1-mm mesh screen (Cyclone Mill, UDY Mfg., Fort Collins, CO). Each sample was scanned by near-infrared reflectance spectroscopy. A FOSS 5000 scanning monochromator (FOSS, Danmark) was used for the collection of the reflectance measurements (log 1/R) between 1100 and 2500 nm, recorded at 2-nm intervals. The Coefficients of determination (R^2) were 0.9589 for CP, 0.9573 for RUP, 0.8638 for K, 0.8243 for Ca, 0.7348 for Mg, and 0.6452 for P.

Genotyping

Eighty-five polymorphic SSRs were used for genotyping the alfalfa panel [29, 30]. Genotyping analysis have been described by Qiang et al. [31]. The genotype data of 336 alfalfa genotypes were deposited in Additional file 4.

Data analysis

The analysis of variance (ANOVA) of all phenotypic data was conducted using the general linear model in the SAS 8.02 [32]. The broad-sense heritability was calculated as $H = \sigma_g^2/ (\sigma_g^2 + \sigma_e^2/n)$, where σ_g^2 is the genotypic variance, σ_e^2 is the environmental variance, and n is the number of the replications.

Genotypic data were filtered using a 5% cutoff value for minor allele frequency using the Tassel v2.1 software [33]. The association analysis was conducted using Tassel v2.1 based on three models, namely general linear model (GLM), Q and the compressed mixed linear model (MLM, Q + K) [34]. The association analysis was performed separately for each year's phenotypic data, while the comparison of the three models was done using the mean values of 3 years' data. The Bayesian model-based program STRUCTURE 2.2 [35] was used to infer the population structure (Q) which was described in the previous report [31]. The kinship matrix (K) was calculated using SPAGeDi software [36]. The markers were identified as significantly associated with traits at a significant level of $P < 0.005$.

Additional files

Additional file 1: List of associated SSR alleles of six studied traits in 3 years. (XLSX 109 kb)

Additional file 2: The information about Mean monthly temperature, and precipitation at the experimental location in 3 years. (TIFF 1750 kb)

Additional file 3: Accession no. origin, improvement status, cultivar name, and No. of genotypes sampled of 336 alfalfa genotypes. (XLSX 12 kb)

Additional file 4: The genotype data of 336 alfalfa genotypes. (XLSX 497 kb)

Jia *et al. BMC Plant Biology* (2017) 17:97

Page 7 of 7

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