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Unlocking the genomic regions associated with seed protein contents in Turkish common bean germplasm through genome-wide association study

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Abstract: Human body needs sufficient quantity of protein on daily basis for the normal functioning. Dietary protein in sufficient quantity is becoming a key issue for huge population of the world particularly in developing countries. Present investigation aimed to explore the phenotypic variation and to investigate the genomic regions associated with seed protein contents in Turkish common bean germplasm collected from 19 provinces. Field experiments were conducted according to augmented block design at two locations (Bolu and Sivas) for two consecutive years (2017 and 2018). Analysis of variance revealed highly significant environmental effects ($p < 0.05$) on seed protein contents within environments, while genotype by environment interaction revealed nonsignificant effects. Overall mean protein contents were 36.36%, while Balikesir-3 and Hakkari-38 yielded minimum (31.64%) and maximum (40.70%) protein contents. Among 19 provinces, accessions from Hakkari and Erzincan provinces disclosed maximum and minimum mean protein contents respectively. Scatter plot between seed protein contents and plant height divided the accessions according to their protein contents and plant height. A total of eight stable accessions were selected and can be recommended for future breeding activities. The implemented constellation plot separated the studied germplasm into two populations on the basis of their protein contents. Genotyping by sequencing resulted 7900 DArTseq markers was used for marker-trait association and a total of 11 markers showed significant association for protein contents. During this study, DArT-3365703 marker was identified in both locations (Sivas and Bolu) during 2017 and might be used for future common bean breeding. Physical map revealed the presence narrow regions between identified markers on Pv02 and Pv05. Therefore, these regions should be considered for future breeding activities. We are confident that results of present study will be helpful for marker-assisted breeding of common bean.

Key words: *Phaseolus vulgaris*, food legume, genomic regions, GWAS, Turkey ctor

1. Introduction

It has been estimated that global demand for food will be more than double between 2010 and 2050 due to rapidly growing population, variations in food preferences and increase in urbanization (Gacek et al., 2018). Protein has been found one of the most deficient macronutrient and the demand for high nutritious food with enough quantity is needed to feed this and upcoming generations (FAO, 2013). According to FAO, approximately a global population of 843 million facing hunger problem and nearly one billion have inadequate protein intake (Wu et al., 2014). Human body requires protein on daily basis for its normal functioning and intake of protein deficient food results in several complications like kwashiorkor, marasmus, impaired mental health and weak immune system (Khan et al., 2017). These nutritional deficiencies highlighted the immediate needs to investigate the sustainable ways to combat with food security problems especially the availability of highly nutritious food (Wu et al., 2014). It is reported that nearly 76% of world population relies on plants to meet their daily protein requirements (FAO, 2014). However, climate change is threating the agriculture production system continuously and there is a need to produce nearly 60%–110% more food in order to meet the food demands in 2050 (Tilman et al., 2011). To combat above stated issues, there is a need to utilize various plant breeding and biotechnological approaches to develop climate resilience cultivars having better quality and production.

Plant breeding community made significant efforts for the development of new cultivars after the development and advancement in molecular markers and sequencing technologies (Nadeem et al., 2018a). Scientific community developed various molecular marker systems according to their resources, application and most of these markers were PCR based. However, advancements in next generation technologies (NGS), genotyping by sequencing (GBS) emerged as promising genomic approach for

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the identification of genetic diversity and marker-trait association (Ali et al., 2020; Mogga et al., 2018). Diversity Array Technology Pty Ltd (DArT, Canberra, ACT, Australia), a microarray-based sequence-independent ultrahigh-throughput marker technology was developed in 2001 (Jaccoud et al., 2001). Diversity array technology (DArT) is a DNA hybridization-based method and can result thousands of polymorphic markers in a single assay (Wenzl et al., 2004). DArTseq markers based on GBS technology have been widely utilized for the identification of genetic diversity and marker-trait association (Ali et al., 2020; Mogga et al., 2018).

Characterization of genetic resources is considered starting point for the plant breeding activities as it serves as a source of novel variations that can be later used for the marker-assisted breeding of crops (Nadeem et al., 2020a,b). Quantitative trait loci (QTL) mapping and genome-wide association studies (GWAS) are two important approaches used by the scientific community for the investigation of genomic regions associated with traits of interest (Nadeem et al., 2020a). However, GWAS emerged as more trustable and high throughput by overcoming the limitation in QTL mapping (Korte and Farlow, 2013). Importance of this approach can be understandable from this statistics that from 2005 to 2018, nearly 3639 GWAS studies have been documented for the identification genetic variant having association with trait of interest (Mills and Rahal, 2019).

Food legumes are serving a great source of dietary protein for human being and they are very important pillar of sustainable agriculture and world food security (Khazaei et al., 2019; Stagnari et al., 2017). Besides serving a source of protein for more than one billion people worldwide, food legumes are also contributing in environmental and economic benefits (Khazaei et al., 2019). Among various food legumes, common bean (*Phaseolus vulgaris* L.) is considered "grain of hope" by serving a source of food for millions of people all around the world (Nadeem et al., 2020a,b). Common bean is rich in protein, carbohydrate, minerals, vitamins and antioxidants that are necessary for the normal functioning of human body (Celmeli et al., 2018). According to FAO (2016), common bean production was 23 million tons in 2010 that reached to 26 million tons in 2016.

A good number of studies have documented aiming to explore the protein content variations in common bean (Katuuramu et al., 2018; Kocira et al., 2017; Guzmán-Maldonado et al., 2000). A limited number of studies have been conducted to investigate the QTL/linked markers associated with protein contents in the seeds of common bean. Casanas et al. (2013) aimed to explore QTL associated with seed chemical contents and reported that QTLs associated with protein content is present on Pv05 and Pv07. Pérez-Vega et al. (2010) identified a total of 13 loci underlying seed protein contents in a RIL population of common bean. To the best of our knowledge, only one study has been documented for the investigation of genetic basis associated with seed protein content using GWAS. Katuuramu et al. (2018) reported a total of five SNPs having association for this trait. By considering the importance of protein for human health, there is a need to conduct more studies to investigate genetic basis associated with protein contents. Keeping in view, the present study was conducted under multiple environment/location to explore the phenotypic diversity of seed protein contents and to investigate the genomic regions associated with seed protein contents in Turkish common bean germplasm.

2. Materials and methods

2.1. Plant material and field experimentation

Plant materials used in this study consist of 182 common bean landraces collected from 19 provinces of Turkey and six commercial cultivars (Akman, Göynük, Karacaşehir, Önceler, Göksun, and Akdağ). The detailed information about plant material can be found from our previously published studies (Nadeem et al., 2018b, Nadeem et al., 2020a). Augmented block design was opted as a sowing plan during the present investigation. Field experiments were conducted at Bolu and Sivas provinces of Turkey. Two experimental years in Bolu (2017 and 2018) and two in Sivas (2017, 2018) were taken as four environments for analytical purposes, as this is a common practice in agricultural experimentations (Gomez and Gomez, 1984). Detailed information about experimental design, sowing time and adopted cultural practices during this study can be found from our previous published studies (Nadeem et al., 2020a,b).

2.2. DNA extraction and genotyping for DArTseq markers

Genomic DNA was isolated from two-week-old seedlings by following CTAB protocol of Doyle and Doyle (1990) and a specific protocol suggested by Diversity Arrays Technology (available at https://www.diversityarrays.com/orderinstructions/plant-dna-extraction-protocol-for-dart/). DNA concentration was maintained as 50 ng μL^{-1} and DNA samples were processed to Diversity Array Technology Pty, Ltd. (Bruce, Australia= (http://www.diversityarrays.com/) for genotyping by sequencing analysis (GBS). Detailed information about GBS analysis for DArTseq markers for studied germplasm can be found from our previously published study (Nadeem et al., 2018b).

2.3. Determination of seed protein contents

To calculate seed protein contents, firstly, seeds of each accession were grounded and total seed nitrogen was determined through Kjeldahl method (Bremner, 1965).

Seed protein contents were calculated as percentage (%) by multiplying the total seed nitrogen values with conversion factor of 6.25 as per the AOAC (1984) methodology.

2.4. Statistical analysis

An online software developed by Rathore et al. (2004) was used for the statistical inferences. The analysis of variance (ANOVA) was calculated within the environments first and adjusted means were derived. These adjusted means were later used to calculate the ANOVA across the environments. Scatter plot was constructed between seed protein contents and plant height through the software XLSTAT (www.xlstat.com). Plant height data was taken from our previously published study (Nadeem et al., 2020). Most stable accessions for protein contents were investigated through online STABILITYSOFT software (Pour-Aboughadareh et al., 2019). The JMP 14.1.0 statistical software (2018, SAS Institute Inc., Cary, NC, USA) was used to construct constellation plot for 188 common bean accessions.

2.5. Investigation of population structure, marker-trait association and putative genes for protein contents

Population structure of Turkish common bean germplasm was performed and Q-matrix for each sample were derived. Structure analysis for studied germplasm was performed under previously published study by Nadeem et al. (2018b). TASSEL 5.0.5 (https://tassel.bitbucket.io) software was used for the investigation of kinship (K) matrix according to Bradbury et al. (2007). Mixed linear model (MLM, Q + K) approach was used to uncover the genomic regions associated with protein contents in common bean. FDR and Bonferroni thresholds were used and markers having $p = 0.01$ were evaluated as significantly associated markers for protein contents. Manhattan plot were constructed through R 3.4.1 statistical software (http://www.r-project.org/) using qq-man R Package (Turner, 2014). A physical map based on chromosome and physical base pair distance between the SNP markers associated with protein contents was developed through R 3.4.1 statistical software. Sequences of investigated DArTseq markers were BLAST in Phytozome V.12.1 (http://phytozome.jgi.doe.gov/pz/portal.html) and legume information system (LIS: https://legumeinfo.org/) databases to identify the putative genes.

3. Results

The analysis of variance for within environments revealed highly significant effects ($p < 0.05$) of environments on seed protein contents. However, analysis of variance for across the environments revealed nonsignificant effects of both genotype and genotype by environment interaction (GEI) (Table 1). Variations of protein contents in the seeds of all common bean accessions used in this study are provided in Table 2. During 2017, protein contents in Bolu ranged from 25.75 to 40.50 for Balikesir-3 and Hakkari-38, respectively, while mean protein contents during 2017 at Bolu were 33.89%. During 2018 at Bolu, minimum and maximum protein contents ranged from 28.94% to 50.13% in Elazığ-30 and Muş-18, respectively, while 37.78% was mean protein content. For the Sivas location during 2017, mean protein content was 34.04%, while maximum (42.63%) and minimum (27.38%) protein contents were reflected by Malatya-51 and Elazığ-10, respectively. During 2018 at the same location, mean protein content was 39.74%, while Bitlis-115 and Bitlis-76 reflected maximum (49.44%) and minimum (34.38%) protein contents. When data of all four environments was combined, overall mean protein content in the studied germplasm was 36.36%, while Balikesir-3 and Hakkari-38 yielded minimum (31.64%) and maximum (40.70%) protein contents. Frequency distribution analysis revealed normal distribution of protein contents in both locations and environments (Figure 1).

Diversity in protein contents was also observed at provinces level and it was observed that accessions belonging to Hakkari province have maximum mean protein contents, while minimum protein contents were observed in the accessions from Erzincan province (Table 3). Scatter plot was constructed between seed protein contents and plant height, which divided the accessions according to their height (Figure 2). Stability analysis revealed a total of eight most stable accessions for protein contents (Table 4). The implemented constellation plot separated the studied germplasm into two populations on the basis of their protein contents (Figure 3).

3.1. Genomic regions and putative genes for seed protein contents

A total of 11 DArTseq markers showed statistically significant association with seed protein contents for all

Table 1. Summary of across the environments analysis of variance in Turkish common bean germplasm.

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Genotype	187	2030.94	10.86064	0.656252	0.99937
GxE	188	3196.477	17.00254	1.027374	0.409842
Residuals	376	6222.614	16.5495	NA	NA

Df: Degree of freedom, Sum Sq: Sums of squares, Mean Sq: Mean Squares, Pr: Probability.

Table 2. Protein contents (%) in the seeds of Turkish common bean germplasm.

Accessions	Bolu17	Sivas17	Bolu18	Sivas18	Mean
Bingöl-1	32.31	30.13	36.88	40.56	34.97
Bingöl-6	35.18	33.75	37.44	39.56	36.48
Bingöl-7	34.13	33.75	35.25	42.31	36.36
Bingöl-11	35.00	31.38	38.13	40.94	36.36
Bingöl-16	34.31	32.13	41.25	39.00	36.67
Bingöl-18	32.38	33.31	38.25	40.19	36.03
Bingöl-25	32.13	33.00	38.31	42.38	36.45
Bingöl-33	32.75	32.13	38.06	40.38	35.83
Bingöl-36	35.31	34.38	36.31	37.94	35.99
Bingöl-44	31.13	29.69	35.50	39.94	34.06
Bingöl-45	35.00	32.06	37.00	39.38	35.86
Bingöl-52	30.31	29.63	38.50	39.31	34.44
Bingöl-53	33.75	31.81	41.75	41.63	37.23
Bingöl-58	35.44	31.94	36.63	43.19	36.80
Bingöl-60	30.44	27.69	37.63	40.38	34.03
Bingöl-61	32.63	28.25	40.94	37.56	34.85
Bingöl-63	31.25	29.81	29.88	38.19	32.28
Bingöl-65	32.50	32.13	39.69	36.63	35.24
Hakkari-7	34.25	32.56	41.00	40.63	37.11
Hakkari-11	36.94	32.38	39.63	40.00	37.24
Hakkari-12	33.56	33.19	45.25	40.38	38.09
Hakkari-13	31.13	30.56	39.31	37.69	34.67
Hakkari-16	35.38	33.69	41.25	40.31	37.66
Hakkari-20	38.75	40.00	40.31	40.69	39.94
Hakkari-23	36.25	36.81	37.19	41.63	37.97
Hakkari-28	35.31	34.63	31.13	41.63	35.67
Hakkari-31	30.75	39.69	32.88	41.63	36.24
Hakkari-37	32.19	32.69	37.50	39.31	35.42
Hakkari-38	40.50	38.75	38.94	44.63	40.70
Hakkari-39	35.31	32.06	38.31	37.19	35.72
Hakkari-43	30.06	35.00	36.00	39.88	35.23
Hakkari-44	33.75	32.25	38.13	39.00	35.78
Hakkari-51	38.13	38.69	38.13	38.81	38.44
Hakkari-55	36.06	39.13	35.19	40.13	37.63
Hakkari-59	35.50	32.13	37.63	40.06	36.33
Hakkari-63	32.50	36.63	41.31	38.88	37.33
Hakkari-65	36.94	37.06	38.56	39.38	37.98
Hakkari-69	35.94	35.94	37.31	39.63	37.20
Hakkari-71	34.50	33.13	42.13	40.31	37.52
Hakkari-76	33.25	31.94	45.13	38.13	37.11
Tokat-83	30.00	27.40	38.81	39.81	34.00

Table 2. (continue)

Accessions	Bolu17	Sivas17	Bolu18	Sivas18	Mean
K.Maraş-92	34.31	32.13	42.44	39.19	37.02
Bitlis-5	36.88	36.13	39.31	40.00	38.08
Bitlis-14	31.81	31.00	43.56	42.13	37.12
Bitlis-16	31.25	33.19	39.56	39.19	35.80
Bitlis-22	32.44	35.00	38.81	37.44	35.92
Bitlis-25	32.31	37.94	37.00	42.19	37.36
Bitlis-35	32.38	32.13	36.25	37.38	34.53
Bitlis-40	35.56	36.38	41.31	36.13	37.34
Bitlis-46	34.94	35.56	33.50	36.25	35.06
Bitlis-48	33.75	32.81	29.44	37.69	33.42
Bitlis-53	37.50	37.25	41.38	42.50	39.66
Bitlis-66	37.44	33.63	39.25	36.19	36.63
Bitlis-69	34.38	35.56	36.25	36.50	35.67
Bitlis-71	32.63	35.69	37.75	34.38	35.11
Bitlis-79	36.50	36.63	37.13	35.50	36.44
Bitlis-81	37.13	37.75	39.06	36.31	37.56
Bitlis-90	38.13	33.13	41.31	35.25	36.96
Bitlis-94	30.25	33.81	39.69	41.31	36.27
Bitlis-97	37.44	33.13	35.13	43.38	37.27
Bitlis-103	40.06	41.69	38.31	38.81	39.72
Bitlis-105	36.13	35.94	37.63	43.06	38.19
Bitlis-111	31.94	32.38	35.44	36.44	34.05
Bitlis-114	36.13	32.81	35.69	35.50	35.03
Bitlis-115	30.63	36.31	33.56	49.44	37.49
Bitlis-117	39.38	34.25	36.25	38.38	37.06
Bitlis-118	31.94	29.50	35.06	38.25	33.69
Bitlis-119	35.00	34.63	34.50	35.56	34.92
Bitlis-120	33.88	31.63	32.94	42.88	35.33
Bitlis-121	38.63	37.50	35.69	45.94	39.44
Bitlis-124	36.94	35.50	37.38	42.00	37.95
Malatya-3	35.56	38.00	37.69	36.69	36.98
Malatya-13	37.63	38.13	31.81	39.00	36.64
Malatya-14	39.50	35.50	39.88	41.13	39.00
Malatya-18	33.63	33.13	39.81	41.38	36.99
Malatya-25	31.88	30.00	34.06	39.19	33.78
Malatya-28	34.75	37.50	39.50	38.25	37.50
Malatya-32	35.50	35.31	38.63	42.06	37.87
Malatya-33	31.94	31.63	36.69	42.94	35.80
Malatya-45	37.06	31.81	40.25	41.88	37.75
Malatya-50	38.25	39.44	35.00	39.19	37.97
Malatya-51	39.50	42.63	34.44	42.94	39.88
Malatya-52	33.50	36.31	38.44	40.13	37.09

Table 2. (continue)

Accessions	Bolu17	Sivas17	Bolu18	Sivas18	Mean
Malatya-59	31.88	32.94	35.38	38.81	34.75
Malatya-71	34.25	36.44	35.81	42.50	37.25
Tunceli-1	35.19	36.38	36.56	42.06	37.55
Tunceli-5	32.69	33.38	36.69	43.13	36.47
Tunceli-11	30.50	31.00	34.69	45.31	35.38
Van-1	30.88	31.56	38.38	48.19	37.25
Van-11	35.63	37.63	36.88	44.63	38.69
Van-13	34.00	32.38	36.38	44.69	36.86
Van-17	35.94	33.13	35.38	48.56	38.25
Van-19	30.56	37.31	35.44	42.06	36.34
Van-25	29.81	32.63	35.81	45.31	35.89
Van-27	34.31	35.00	35.25	42.25	36.70
Van-29	31.44	31.31	35.13	45.50	35.84
Van-33	30.63	33.06	40.50	40.38	36.14
Van-36	35.06	33.38	35.56	36.63	35.16
Van-42	35.00	32.06	37.69	38.81	35.89
Van-47	33.19	33.00	32.38	40.25	34.70
Van-51	38.25	34.69	38.75	40.81	38.13
Van-64	38.94	42.00	37.06	39.38	39.34
Van-65	28.13	35.63	36.81	39.00	34.89
Van-68	34.25	36.25	39.00	40.19	37.42
Van-59	38.13	41.25	35.13	37.88	38.10
Elazığ-2	34.06	37.50	32.69	38.31	35.64
Elazığ-7	30.31	33.19	37.00	36.69	34.30
Elazığ-9	34.50	35.25	34.13	37.44	35.33
Elazığ-10	28.63	27.38	34.63	38.19	32.21
Elazığ-14	33.00	37.06	40.19	40.13	37.59
Elazığ-16	31.31	29.25	35.63	41.06	34.31
Elazığ-25	34.94	34.13	36.38	40.00	36.36
Elazığ-27	32.19	31.94	37.31	42.38	35.95
Elazığ-29	34.94	32.06	37.00	41.06	36.27
Elazığ-30	33.75	32.25	28.94	38.38	33.33
Elazığ-34	32.44	32.50	43.31	35.25	35.88
Elazığ-36	34.81	36.19	36.94	39.81	36.94
Elazığ-39	32.63	32.63	42.50	38.13	36.47
Muş-1	33.44	35.75	37.56	38.75	36.38
Muş-2	36.88	39.94	38.50	38.63	38.49
Muş-7	36.31	36.44	37.69	41.31	37.94
Muş-10	33.69	34.19	39.25	39.81	36.74
Muş-15	32.06	32.88	39.25	37.31	35.38
Muş-18	35.25	33.94	50.13	37.13	39.11
Muş-22	30.00	38.31	34.75	38.75	35.45

Table 2. (continue)

Accessions	Bolu17	Sivas17	Bolu18	Sivas18	Mean
Muş-27	31.25	34.75	36.25	40.06	35.58
Muş-28	38.44	40.00	39.81	38.63	39.22
Muş-34	38.13	41.38	34.19	39.31	38.25
Muş-39	33.13	34.56	36.06	41.81	36.39
Muş-41	35.56	35.13	39.81	37.13	36.91
Muş-42	33.00	37.56	41.06	37.63	37.31
Muş-43	33.13	34.31	46.31	39.31	38.27
Muş-46	34.44	35.31	37.00	38.13	36.22
Muş-48	34.63	30.50	30.56	39.13	33.70
Muş-49	30.81	32.69	36.94	35.63	34.02
Muş-50	33.50	31.00	35.44	38.50	34.61
Muş-51	29.69	27.56	39.00	37.56	33.45
Muş-52	30.25	34.50	39.19	41.69	36.41
Muş-53	36.13	36.56	37.50	40.75	37.74
Sivas-3	29.44	30.13	39.19	38.31	34.27
Sivas-4	31.38	31.58	37.56	39.81	35.08
Sivas-7	36.00	34.31	36.25	39.69	36.56
Sivas-12	33.06	35.63	43.31	40.06	38.02
Sivas-13	31.25	36.56	41.81	41.81	37.86
Sivas-16	33.13	34.31	38.75	39.31	36.38
Sivas-17	31.06	34.25	35.44	40.94	35.42
Sivas-18	33.38	35.44	34.19	39.38	35.60
Sivas44	40.06	38.38	36.25	40.75	38.86
Sivas62	32.63	32.56	37.44	40.44	35.77
Sivas68	28.63	31.25	37.75	40.94	34.64
Sivas69	31.06	33.00	36.38	39.38	34.95
Sivas-70	35.56	35.56	38.88	38.25	37.06
Bilecik-1	31.25	36.19	46.19	39.00	38.16
Bilecik-2	36.88	35.50	38.75	40.19	37.83
Bilecik-6	34.44	36.25	37.81	39.06	36.89
Bilecik-7	34.19	31.69	39.50	39.31	36.17
Bilecik-8	36.06	33.00	37.50	39.19	36.44
Bilecik-10	31.44	34.13	37.50	39.56	35.66
Balıkesir-3	25.75	30.81	31.75	38.25	31.64
Balıkesir-4	33.13	30.19	38.06	40.13	35.38
Balıkesir-5	32.63	32.13	41.81	39.06	36.41
Balıkesir-6	31.25	28.25	38.44	40.69	34.66
Balıkesir-17	35.63	33.94	39.00	39.19	36.94
Balıkesir-18	31.63	29.69	40.56	44.69	36.64
Balıkesir-19	29.94	37.06	32.31	39.50	34.70
Balıkesir-20	36.38	41.00	34.94	43.81	39.03
Düzce-1	36.69	32.13	37.13	39.63	36.39

Table 2. (continue)

Accessions	Bolu17	Sivas17	Bolu18	Sivas18	Mean
Düzce-9	33.25	33.31	40.25	36.94	35.94
Yalova-13	33.38	33.63	34.19	36.50	34.42
Yalova-20	33.69	33.88	40.50	38.63	36.67
Yalova-21	33.44	32.13	39.50	37.19	35.56
Erzincan-1	33.06	36.31	39.31	35.44	36.03
Erzincan-3	34.00	32.75	38.88	39.56	36.30
Erzincan-4	34.31	31.88	34.13	37.56	34.47
Erzincan-5	28.44	30.50	40.63	34.94	33.63
Bursa-1	30.88	29.13	40.13	39.81	34.99
Bursa-22	31.88	36.81	38.25	37.31	36.06
Dermasyon	35.06	34.81	38.56	39.25	36.92
Derinkuyu	32.69	31.44	44.81	35.63	36.14
Civril-Bolu	37.75	36.94	35.31	36.56	36.64
Bolu-Göynük	36.88	30.88	45.81	38.38	37.99
Moralaca	33.75	34.44	41.63	36.44	36.56
Akman ×	35.63	33.95	38.34	41.47	37.35
Göynük ×	32.92	28.41	37.62	41.61	35.14
Karacaşehir ×	33.41	33.17	39.30	39.59	36.37
Onceler×	33.18	32.73	37.69	40.00	35.90
Göksun×	31.26	30.74	37.75	39.94	34.92
Addag×	32.80	32.16	37.94	39.81	35.68

× Commercial cultivars

four environments (Table 5). During both years of study (2017–2018) in Bolu, a total of six markers (3 markers for each environment) disclosed significant association with protein contents (Figures 4 and 5). A total of three markers (two markers in 2017 and one in 2018) showed significant association for protein contents in Sivas location for both environment (Figures 6 and 7). When the data of all four environments was combined, a total of two DArTseq marker showed significant association for the protein contents (Figure 8). A total of 11 putative genes (one for each identified marker) were predicted from the sequences reflecting homology to identified DArTseq markers (Table 5). A physical map was developed that revealed narrow regions between the DArTseq markers on Pv02 and Pv05 (Figure 9).

4. Discussion

Dietary protein in sufficient quantity is becoming a key issue for future food security. Beside animal resources, plant based food significantly contributing to provide sufficient quantity of protein required for normal functioning of the body (Chardigny and Walrand, 2016). Considering the significance of protein contents in global food as well as nutritional security, improving the quality and protein content in the most consumed part (seeds) of various crop is now becoming the most challenging task in molecular breeding and genomics research (Upadhyaya et al., 2016). Therefore, it is very important to characterize the germplasm for the investigation of genetic variations that can be helpful in breeding activities (Nadeem et al., 2021; Shimira et al., 2021; Ghomi et al. 2021; Nadeem, 2021). Common bean is considered "grain of hope" because of having higher contents of protein, mineral, vitamins and antioxidants. Current study disclosed the phenotypic variations of protein contents in the seeds of Turkish common bean germplasm and to unlock the genomic regions associated with this trait.

Analysis of variance (ANOVA) revealed highly significant effects ($p < 0.05$) of environments on the seeds protein contents for all four environments. Flores-Sosa et al. (2020) disclosed significant differences ($p \leq 0.05$) in protein and amino acid content between the populations. Ceyhan et al. (2008) found significant difference ($p < 0.01$) for protein content between years and cultivars. The calculated genotype by environment interaction (GEI) revealed nonsignificant effects of both genotype and GEI. Razvi et al. (2011) revealed that genotype by interaction has no significant effect on protein contents in the seeds of common bean and supported the findings of this study. During this study, a good range of variations of protein contents was observed for each environment (Table 2). Overall protein contents (mean of all four environments) ranged between 31.64% and 40.70% during this study. Mean and range of protein contents found in this study were much higher than the report of Ceyhan et al. (2008) where they identified protein content in a range of 21.40%–27.29%. Ganesan and Xu (2017) stated that protein contents in dry beans range between 20% and 30%. Esteves et al. (2002) found protein contents in a range of 22% and 26%, while Oliveira et al. (2001) reported mean protein values of 19.8% in common bean germplasm. Similarly, Brigide et al. (2014) also reported much lower protein contents (22.24 to 31.59%) than reported in this study. Dostalova (2002) stated that protein composition in the seeds is subjected to different factors such as type of plant material, maturity stage, agrotechnics and weather conditions. Frequency distribution analysis revealed normal distribution of protein contents in both locations and environments (Figure 1).

As the world is facing unprecedented pattern of climatic changes and investigation and subsequent selection of stable genotypes, reflecting superior performance under multiple environment/location is becoming key area of interest for the breeding community (Ahmadi et al., 2015; Vaezi et al., 2018). Previously, various parametric methods have been proposed by the scientists. Most popular and commonly

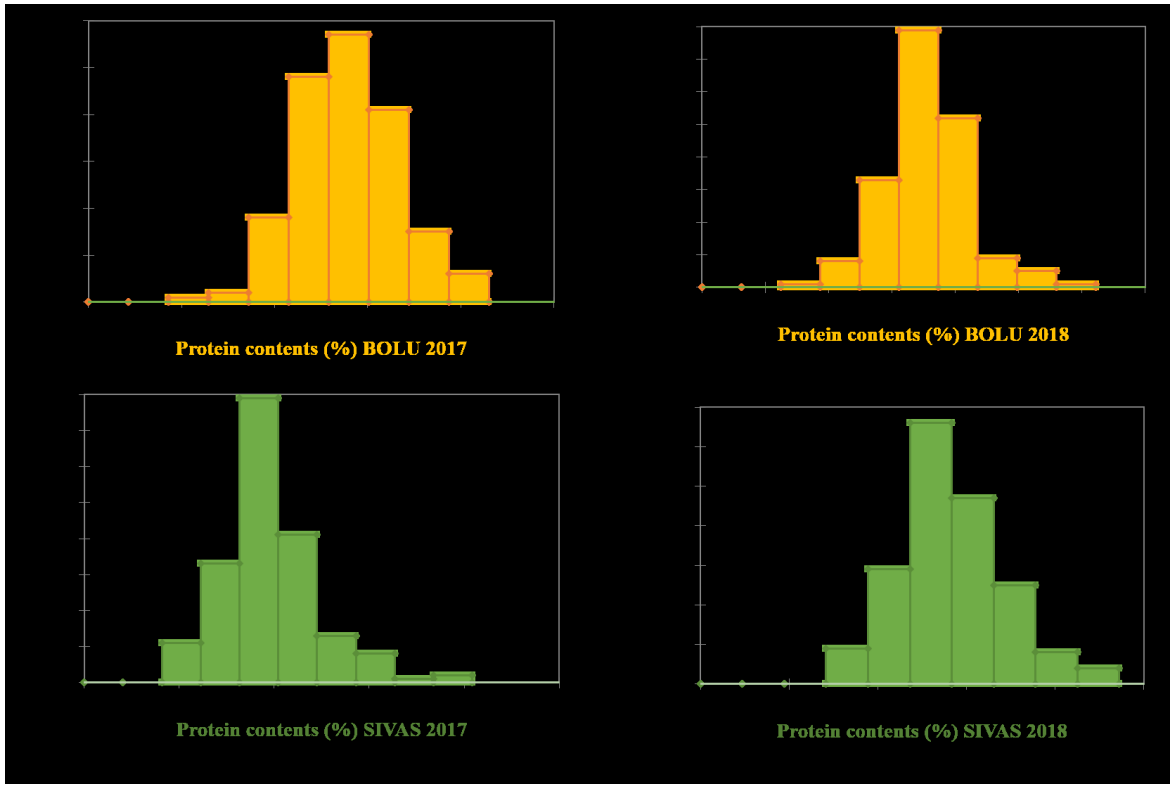

Figure 1. Frequency distribution of protein contents in Turkish common bean germplasm during this study.

Table 3. Provinces based variations of protein contents in the seeds of Turkish common bean germplasm.

Provinces	Minimum	Maximum	Mean	Std. deviation
Bingöl	32.281	37.234	35.55	1.251
Hakkari	34.673	40.703	37.14	1.506
Tokat	31.940	45.130	37.11	1.470
K.Maraş	27.400	39.810	34.00	1.270
Bitlis	33.421	39.719	36.54	1.667
Malatya	33.783	39.876	37.09	1.562
Tunceli	35.375	37.549	36.46	1.087
Van	34.704	39.344	36.80	1.372
Elazığ	32.206	37.593	35.43	1.513
Muş	33.453	39.219	36.55	1.717
Sivas	34.268	38.860	36.19	1.419
Bilecik	35.658	38.157	36.86	0.971
Balıkesir	31.640	39.033	35.67	2.161
Düzce	32.720	38.700	36.16	1.659
Yalova	34.424	36.674	35.55	1.125
Erzincan	33.626	36.297	35.11	1.274
Bursa	31.380	39.190	35.53	1.120
Niğde	33.130	41.690	36.53	1.175
Bolu	36.563	37.987	37.06	0.800
Cultivars	34.922	37.348	35.89	0.883

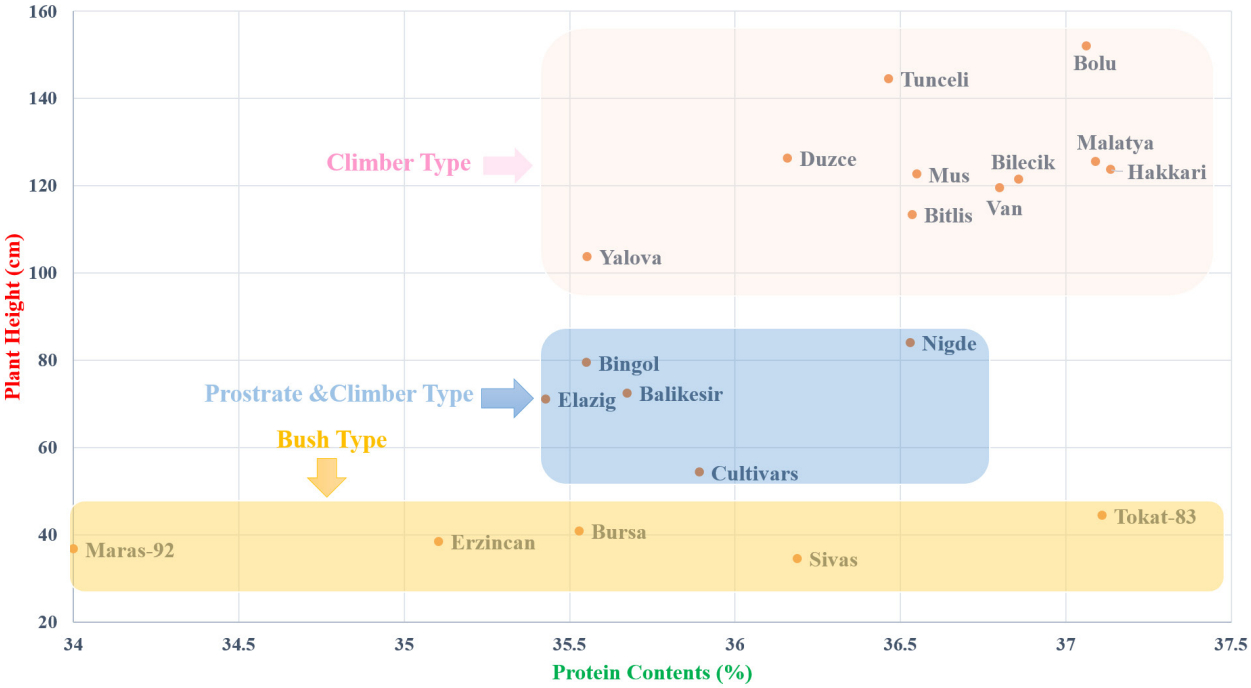

Figure 2. Scatter plot between protein contents and plant height of Turkish common bean germplasm.

Table 4. Most stable common bean accessions regarding protein contents.

Genotype	Protein content	W_i^2	σ^2_i	s^2d_i	b_i
Bitlis-40	34.53	0.484075	0.122128	0.046147	0.919726
Bitlis -66	39.66	0.490027	0.124133	0.047322	0.920295
Malatya-32	37.87	1.158858	0.349474	0.140896	1.083101
Hakkari-37	35.42	1.423779	0.43873	0.032709	1.218652
Sivas-62	35.77	2.946752	0.951847	0.016517	1.336576
Bitlis -111	34.05	1.54619	0.479972	0.026844	0.76687
Onceler	35.90	1.426954	0.4398	0.029585	1.220932
Hakkari-51	38.44	1.505722	0.466338	0.209965	1.037936

W2i: Wricke's (1962) ecovalence, σ^{2i}: Shukla's (1972) stability variance, S^2d_i: Deviation from regression (Eberhart and Russell, 1966), b_i: Linear regression coefficient (Finlay and Wilkinson, 1963).

used methods for the selection of stable genotypes include Wricke's equivalence stability index (W2i; Wricke, 1962), Shukla's stability variance (σ2i; Shukla, 1972), deviations from the regression (S2di; Eberhart and Russell, 1966) and linear regression coefficient (bi; Finlay and Wilkinson, 1963). Among these calculated parameters, preference was given to the Shukla's stability variance (σ2i; Shukla, 1972) for the selection of most stable genotypes. Shukla (1972) concluded that accessions having minimum σ2i reflect maximum stability to the environmental conditions. Therefore, a total of eight most stable accessions were evaluated and can be recommended as a parent for the development of protein-enriched common bean cultivars.

Germplasm used in this study was collected from 19 provinces and accessions belonging to Hakkari province reflected maximum mean protein contents, while minimum protein contents were observed in the accessions from Erzincan province (Table 3). Scatter plot revealed that accessions having minimum plant height or bushy growth habit have less protein contents (Figure 2). For example, it can be seen that accessions from Erzincan, Sivas, Bursa, K. Maraş and Tokat have bushy growth habit. They

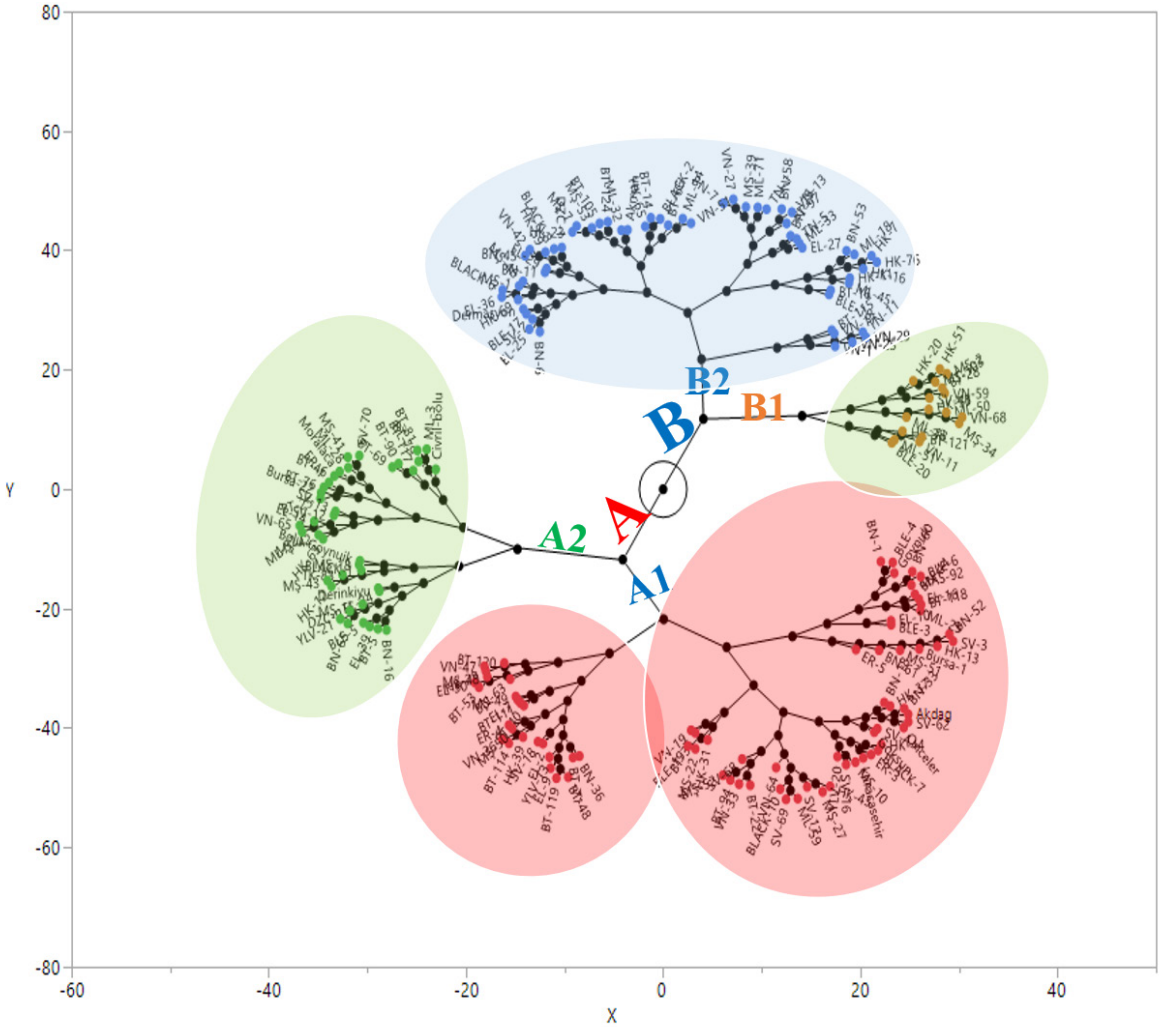

Figure 3. Constellation plot for seed protein contents in Turkish common bean germplasm.

Table 5. Marker-trait association for protein contents in Turkish common bean germplasm.

Environment	No. Markers	Markers	CHR	POS	p-value	R^2	Putative gene
B-17		3374740	5	2930258	7.00E-04	6.79	Phvul.005G031700
		3365703	5	2805088	7.43E-04	7.24	Vigun01g031300
	03	3366179	9	36961270	8.27E-04	6.6	Phvul.009G249100
B-18		3375856	2	7685507	0.00215	5.81	Phvul.005G106800
		3373939	5	27655625	0.00246	5.43	Phvul.008G112400
	03	3367239	2	45921418	0.00358	5.07	Phvul.002G290700
S-17		8178858	4	1347278	9.31E-04	6.51	Vang02g14290
	02	3365703	5	2805088	0.00132	6.58	cicar.ICC4958.Ca_12756
S-18	01	8210415	7	47320728	3.17E-04	8.33	Vigun07g055800
Overall		8671084	2	21210792	5.58E-04	7.35	Phvul.004G085000
	02	3373184	2	22533921	9.51E-04	6.55	Phvul.002G105700

CHR: Chromosome, POS: Position.

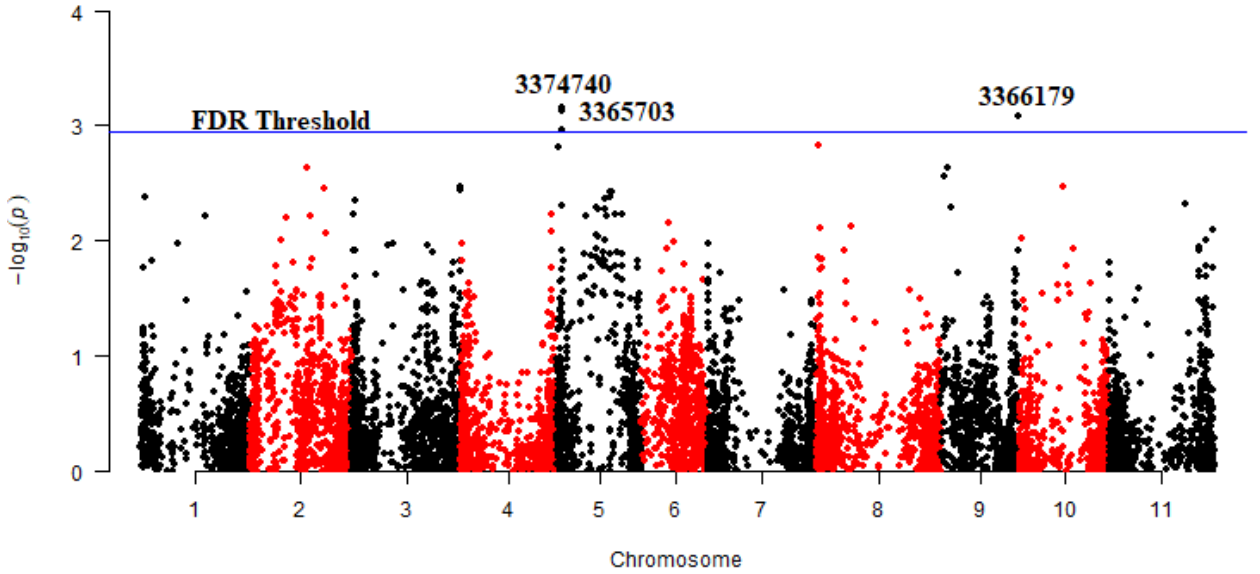

Figure 4. Manhattan plot for protein contents in Turkish common bean germplasm during 2017 for Bolu location.

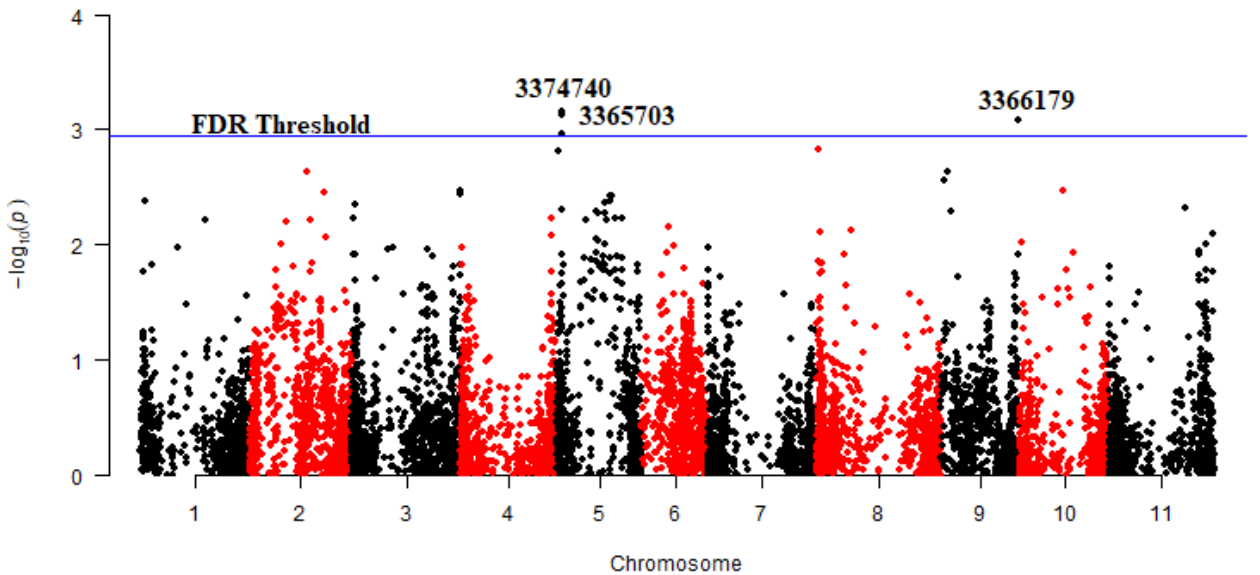

Figure 5. Manhattan plot for protein contents in Turkish common bean germplasm during 2018 for Bolu location.

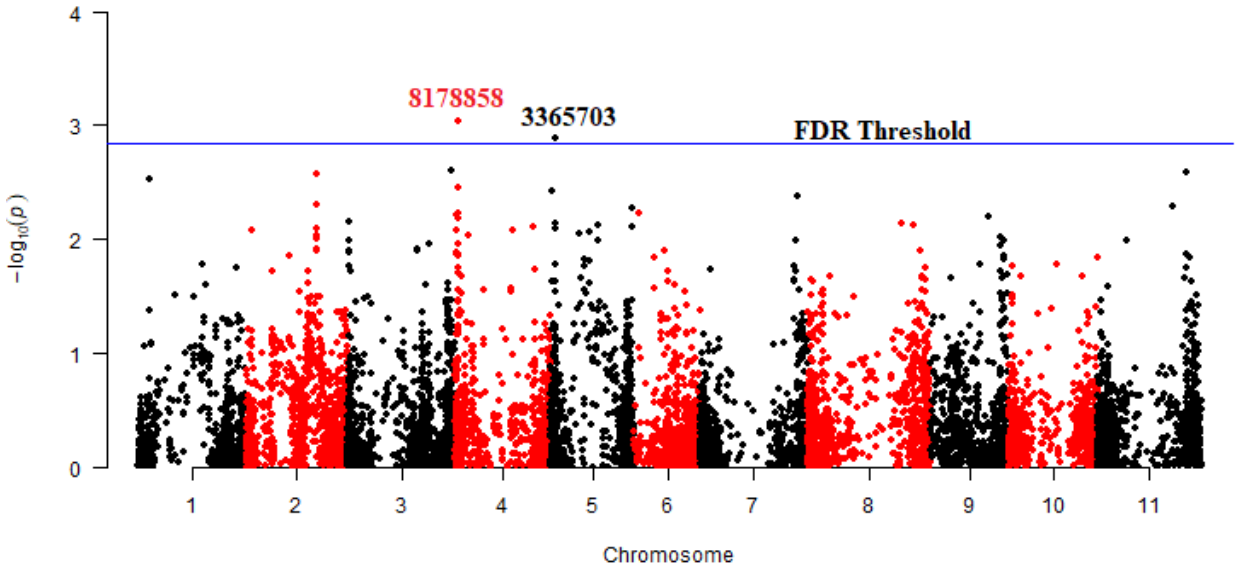

Figure 6. Manhattan plot for protein contents in Turkish common bean germplasm during 2017 for Sivas location.

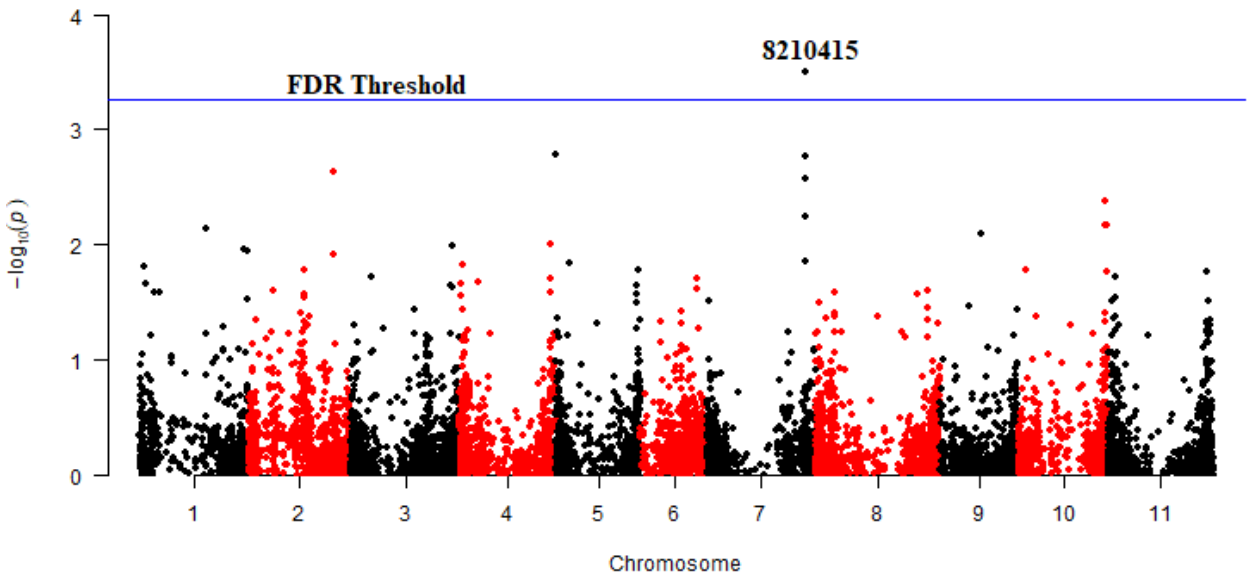

Figure 7. Manhattan plot for protein contents in Turkish common bean germplasm during 2018 for Sivas location.

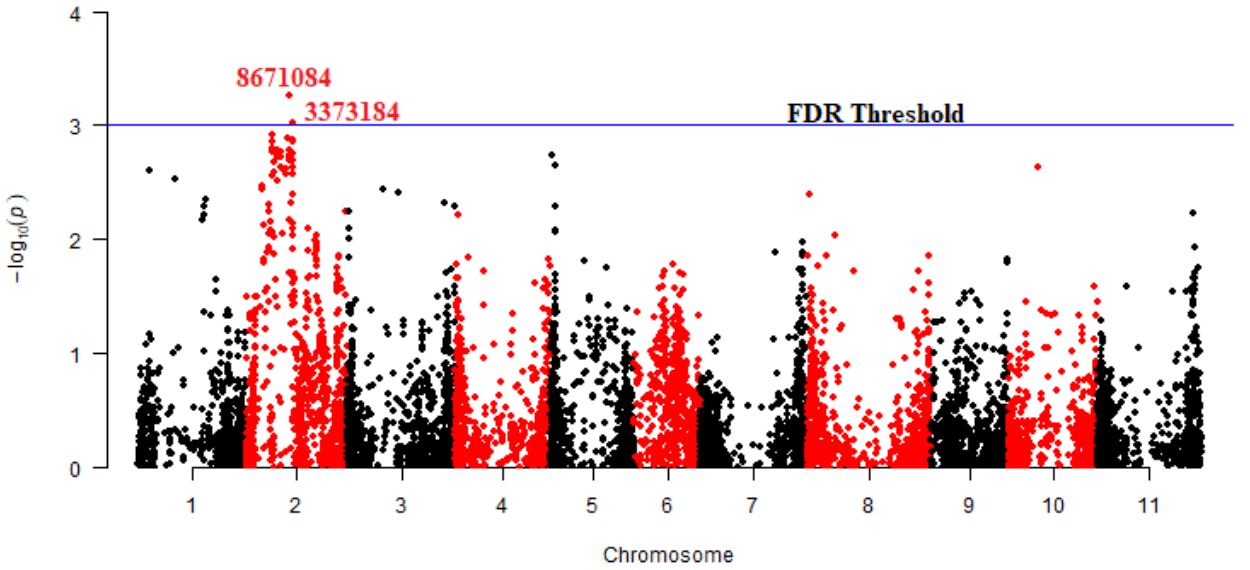

Figure 8. Manhattan plot for protein contents in Turkish common bean germplasm combining all four environmental conditions.

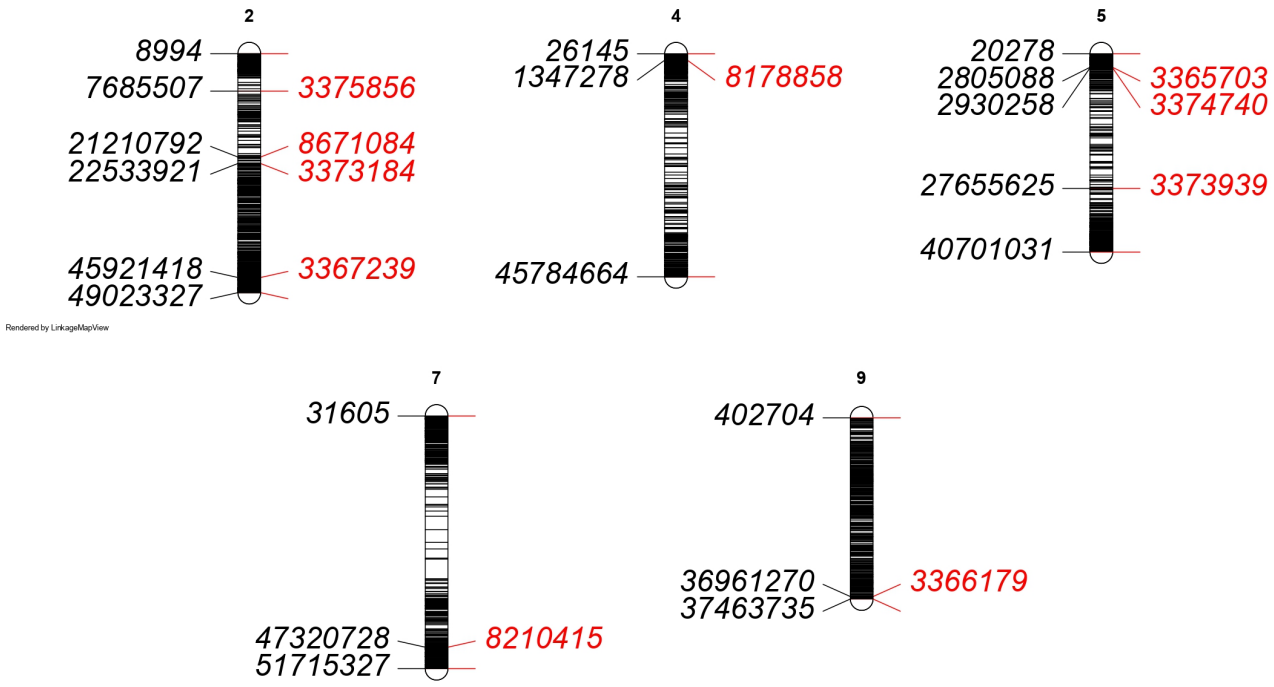

Figure 9. Physical map of chromosomes and physical base pair distance (bp) displaying the significantly associated markers to protein contents.

disclosed minimum protein contents. It was interesting to see that, as the plant height was increasing, an increase in protein contents was also observed. Most of the accessions belonging to East of Turkey i.e. Van, Hakkari, Bitlis, Muş, Bingöl and Tunceli have climber growth habit and reflected higher protein contents. Most probably there are two reasons behind higher protein contents in the seeds of accessions from these provinces; first, climatic conditions of these provinces are very cold and possibly contributed in the accumulation of more protein content under low temperature (Author's perception). Second, growth habit might be a critical factor because bushy genotypes are early maturing, while climbers are late maturing and have more growth cycle. Therefore, having long growth cycle, climber accessions acquire more protein contents compared to bushy ones (Author's perception).

The implemented constellation plot divided germplasm into two population according to their protein contents (Figure 3). Population A was found larger compared to population B and further divided into subpopulation A1 and A2. Subpopulation A1 contains the accessions having protein contents in the range of 32%–36%. Accessions in subpopulation A2 disclosed higher protein contents (36%–39.5%). Population B was also divided into two subpopulations B1 and B2, while subpopulation was found more rich in protein contents (38%–40%) compared to B1 having protein contents in the range of 34%–36%.

A total of 11 DArTseq markers showed significant association for seed protein contents for all four environments (Table 5). Identified markers showed their distribution on Pv02, Pv04, Pv05, Pv07 and Pv09. Maximum number (four) of DArTseq markers were distributed chromosome Pv02. During this study, 3365703 marker was identified in both locations (Sivas and Bolu) during 2017. DArTseq marker 8210415 present on chromosome Pv07 disclosed maximum genetic variations (8.33%), while 3367239 marker present on Pv03 resulted in minimum (5.07%) genetic variations. Localization of DArTseq marker on different chromosomes might be due to the polygenic nature of these traits, influenced by minor genes. Katuuramu et al. (2018) aimed to explore genetic basis associated with nutritional composition-related traits and reported SNPs for protein contents on Pv03 and Pv06 and Pv07. Casanas et al. (2013) used SSR and AFLPs markers and stated that QTL for seed protein are present on Pv05 and Pv07. As maximum number of four and three markers were distributed on chromosomes Pv02 and Pv05, respectively. Physical map disclosed that two DArTseq markers 8671084 and 3373184 were distributed on the chromosomes Pv02 at 21.21 Mbp and 22.53 Mbp, respectively. As both of these markers were present in a narrow region of 1.32 Mbp and both of these markers were identified when data of all environment was combined. Therefore, this region should be considered for future breeding activities of common bean. Similarly, two DArTseq markers (3365703 and 3374740) were also present in a narrow region of 1.25. 3365703 marker was identified in both location during 2017, therefore this region should be also considered very important for future breeding.

The BLAST search against 3374740 marker resulted in Phvul.005G031700 gene which encodes ribosomal protein L27 family protein. Szakony and Byrne (2011) stated that ribosomal protein is essential for ribosome biogenesis and contribute in protein synthesis. Similarly, Szakony and Byrne (2011) also revealed that ribosomal protein L27 is required for growth and multiple developmental processes in *Arabidopsis*. Vigun01g031300 was found putative gene for 3365703 DArTseq marker and encodes Annexin 8 protein. This 3365703 DArTseq marker was found in the exon region of Vigun01g031300 gene. Xu et al. (2016) stated that Annexins are calcium-dependent phospholipid binding proteins that contribute significantly in plant growth and development and stress resistance. Konopka-Postupolska and Clark (2017) comprehensively explored the role of Annexins in plant cell. Phvul.009G249100 was found putative gene for 3366179 marker which encodes for Transducin/WD40 repeat-like superfamily protein. Guerriero et al. (2015) stated that Transducin/WD40 repeat proteins that function as molecular "hubs" and play significantly in various cellular processes such as plant stress and hormone responses. Previous reports showed that WD40 proteins are abundant in plants and play significantly in various process like plant development, cell wall formation, anthocyanin biosynthesis and immunity (Guerriero et al., 2015; Miller et al., 2016). The BLAST search of 3375856 marker's sequence resulted in Phvul.005G106800 which belongs to protein kinase superfamily protein. Stone and Walker (1995) revealed that this superfamily catalyse the reversible transfer of the y-phosphate from ATP to amino acid side chains of proteins. Moreover, they also revealed that almost 1 to 3% of functional eukaryotic genes encode protein kinases, disclosing their significant role in various aspects of cellular regulation and metabolism. The BLAST search against 3365703 marker resulted in cicar.ICC4958. Ca_12756 as a putative gene which encodes actin-binding FH2 (formin homology) protein. A good number of reports has been documented exploring the role of this protein in cell expansion, cell division, morphogenesis or resistance to pathogens (Wasteneys and Galway, 2003; Staiger and Hussey, 2004; Wasteneys and Yang, 2004). Vigun07g055800 was found putative gene for 8210415 marker and encodes receptor-like protein kinase (RLKs). Goff and Ramonell (2007) comprehensively disclosed the functioning of RLKs in plant defense system. They also

stated that RLKs contribute in plant growth, development and hormone perception as well. The BLAST search of 8671084 marker's sequence resulted in the identification of Phvul.004G085000 as a putative gene. This gene encodes for cytochrome P450 superfamily protein. Cytochrome P450 superfamily protein is one of the plant's largest protein family involved in multiple metabolic pathways and promotes growth and development of plants and protecting them from various stresses through multiple biosynthetic and detoxification pathways (Li et al., 2012). Jun et al. (2015) comprehensively explored the role of this family in plant growth and defense system. Phvul.002G105700 was identified putative gene for 3373184 marker and belongs to Basic-leucine zipper (bZIP) transcription factor family protein. Recently, Gai et al. (2020) stated that bZIP protein consist of huge number of transcription factors (TFs) and play a key role in the plant, growth, development and provide resistance to various stresses. Previous studies confirmed that bZIP transcription factor are involved in various biological process like floral transition and initiation, seed maturation and storage protein gene regulation (Walsh et al., 1998; Lara et al., 2003; Shen et al., 2007).

5. Conclusion

This study disclosed the marker-trait association for protein contents very first time using Turkish common bean germplasm. The present investigation was conducted under two provinces of Turkey. Balikesir-3 and Hakkari-38 landraces yielded minimum (31.64%) and maximum (40.70%) protein contents and can be recommended as candidate parents for common bean breeding. A total of 11 DArTseq markers showed their association with seed protein contents from all four environments. Among these 11 markers, DArT-3365703 marker was identified in both locations (Sivas and Bolu) during 2017 and might be used for future common bean breeding. Physical map identified the presence of narrow regions between the markers on Pv02 and Pv05 and these regions should be taken under consideration for future common bean breeding activities.

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