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Association mapping of plant structure and yield traits in faba bean (*Vicia faba* L.)

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Abstract

Background: Faba bean (*Vicia faba* L.) is an important crop with high protein content. Tens of thousands of faba bean accessions are available in germplasm collections throughout the world. Morphological characterization of these materials can enrich these collections and aid in the selection of genotypes for use in breeding programs.

Results: In this study, 26 morphological characters were analyzed for 61 faba bean landraces and 53 cultivars over two seasons in Izmir, Turkey. The genotypes had high diversity for several yield traits including number of pods per plant, dry seed yield, and 100-seed weight. Association mapping was conducted for the morphological characters using 651 alleles from 100 simple sequence repeat (SSR) markers and a general linear model based on the Q matrix. A false discovery rate of 0.20 was used to test the significance of marker–trait associations resulting in 75 loci detected for 20 of the morphological characters ($p \leq 0.001$).

Conclusion: Overall, 44% of the quantitative trait loci (QTLs) were for seed traits, with 24%, 15%, and 17% of QTL identified for vegetative, inflorescence, and pod traits, respectively. The phenotypic data and marker–trait associations generated by this work can help breeding programs in the selection and improvement of faba bean.

KEYWORDS
broad bean (*Vicia faba* L.), diversity analysis, molecular characterization, quantitative trait locus mapping, QTL

INTRODUCTION

Faba bean (*Vicia faba* L.) is an annual legume crop that is planted in the winter in warm-temperate and subtropical countries and in the spring in northern latitudes.[1] Faba bean is an important crop due to its high yield potential and nutritionally dense grains that are rich in protein, carbohydrate, and fiber.[2] Faba bean is low in lipids and an important provider of vitamins and energy with 320 kcal/100 g.[3,4] As a result, faba bean is used as a cheap, healthy source of protein in many developing countries where people cannot afford to buy meat.[5] This crop also has a role in crop rotation due to its symbiosis with nitrogen-fixing bacteria.[1]

According to FAOStat data,[6] world production of faba bean was 5.43 million tonnes in 2019. In 2019, China accounted for 32% of production followed by Ethiopia, the United Kingdom, and Australia with 19%, 10%, and 6% of world production, respectively. Yield varies greatly among regions with the greatest yield in Europe and Asia (over 2 tonnes/ha), while farmers in the Americas only achieve half the productivity obtained in Europe. Germplasm collections are sources of faba bean diversity, which can be leveraged to increase yield and improve other agro-morphological traits. The value of genetic resource collections is greatly enhanced when accessions are evaluated for agronomic, yield, quality, biotic, and abiotic stress traits[7] as this knowledge helps guide the breeder in selecting parents and potential cultivars. Thus, morphological characterization is a

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preliminary and basic requirement for the use of germplasm resource in plant breeding.[8]

Molecular markers can be used to study the genetic diversity and population structure of genetic resources as well as for genetic mapping of various traits. Among these markers are simple sequence repeats (SSRs) that were first discovered by Hamada et al.[9] SSRs can be more informative, variable, and easier to use than other molecular markers and are considered by many as the best marker for revealing polymorphism and preparation of genetic maps.[10] Recently, many SSR markers have been developed in faba bean[11–21] and these markers can be used to study diversity and identify quantitative trait loci (QTLs) linked to morphological or biochemical characters. QTL detection can then be followed by marker-assisted selection (MAS) in breeding programs.

Linkage and association mapping are the two main methods for locating genes or QTLs in a genome. Genetic linkage mapping frequently uses biparental segregating populations. The main limitations of this approach are that only two alleles at any given locus can be studied in diploid biparental crosses and low mapping resolution.[22] In contrast, association mapping, also known as linkage disequilibrium (LD) mapping, utilizes historic recombination events within a population of unrelated individuals for the identification of genetic markers for qualitative or quantitative traits using statistical tools. Association mapping improves mapping resolution, reduces research time, and has the potential to reveal a greater number of beneficial alleles.[23,24] However, this method is limited in its ability to detect QTLs with small effects, those due to rare variants or affected by epistasis. These disadvantages are especially problematic with small populations or genomes that are not fully sequenced.[25] Only a few experiments have been conducted using association mapping for morphological traits in faba bean.[26–28] Ali et al.[26] conducted association mapping in 189 faba bean lines with 175 single nucleotide polymorphism (SNP) and 1147 amplified fragment length polymorphism (AFLP) markers, and many putative QTLs were detected for drought and freezing stress. In three studies by Sallam and coworkers,[27–29] 156 SNP markers generated from a legume model (*Medicago truncatula*) were used for association mapping in 189 faba bean accessions, and putative QTLs were detected for frost tolerance, some morphological, physiological, and yield traits. In addition to association analysis, many more QTL analyses have been performed for morphological traits using biparental mapping populations in faba bean.[30–34] The first attempt was performed by Ramsay et al.[30] using 23 markers [morphological, random amplified polymorphic DNAs (RAPDs), AFLPs, and isozymes] to map morphological and biochemical traits in a backcross F_2 (BCF_2) population. Recently, Aguilar-Benitez et al.[32] identified 12 QTLs for flowering time through a comprehensive linkage analysis in a recombinant inbred line (RIL; Vf6 × Vf27) population of 124 individuals using a previous map including gene markers related to flowering, RAPDs, expressed sequence tag (EST), and SNP markers. Another study conducted by Aguilar-Benitez et al.[34] detected 19 QTLs related to reproductive and morphological traits in the Vf6 × Vf27 RIL population using their previous linkage map.[32] Most recently, Meng-wei et al.[33] carried out a QTL mapping study and identified 98 QTLs for 14 agronomic traits using an integrated genetic linkage map with 6895 SNPs (3324.48 cM).

The aims of this work were to (1) characterize 114 unrelated faba bean genotypes (61 landraces and 53 cultivars) collected from different locations in the world using 26 morphological traits over two growing seasons; (2) study the correlation between these characters; and (3) perform association mapping for the traits using 100 SSR primer pairs. The phenotypic data and marker–trait associations generated by this work are useful resources for the selection and improvement of faba bean.

MATERIALS AND METHODS

Plant materials

Faba bean seeds were provided by the Centre for Genetic Resources, the Netherlands (CGN); Aegean Agricultural Research Institute (AARI, Turkey); the University of Helsinki (Finland); the University of Adelaide (Australia); the Nordic Genetic Resource Center (NGB); and the International Center for Agricultural Research in the Dry Areas (ICARDA, Syria). A total of 114 faba bean genotypes (including 61 landraces and 53 cultivars) from 20 countries were used as plant material (Table S1). Accessions were categorized as landraces or cultivars based on information from the seed banks and from Genesys (https://www.genesys-pgr.org/). Seeds were grown in the field in Gulbahce, Izmir, on the west coast of Turkey with coordinates (38°19′ 47.1″ N and 26°38′ 27.8″ E) during two successive growing seasons (2016/17 and 2017/18). Specific weather data for the two seasons are given in Table S2. Fifteen seeds per accession were sown in single-row plots during the last week of October in 2016 and 2017. Each row was 6 m long and 40 cm wide. There was 30 cm spacing between plants within each row. Spacing between rows was 1 m. The plants were rain fed except when conditions were dry. No fertilizers or pesticides were used during the growing season. The final dry pod harvest was in the last week of May in both 2017 and 2018.

Morphological characterization

Five plants were randomly selected from each genotype in each season to evaluate the 26 morphological characters (14 quantitative and 12 qualitative) as indicated in supplementary materials (Table S3). The quantitative characters were: leaflets per leaf, number of stems per plant, plant height (cm), number of flowers per inflorescence, pod length (cm), number of pods per node, total number of pods per plant, maximum number of ovules per pod, number of seeds per pod, seed fertilization (%), fresh seed water content (%), 100-seed weight (g), and dry seed yield per plant (g). The qualitative characters were leaflet shape, stipule spot pigmentation, stem pigmentation at flowering time, mature stem color, intensity of petal streaks, wing petal color, pod angle, pod shape, pod surface reflectance, seed shape, seed coat color, and hilum color. Characterization was done according to the methods and scales of the faba bean descriptors of the International Board for

Plant Genetic Resources (IBPGR)[35] and is briefly described in Table S3.

Statistical analysis

Means, coefficients of variation (CV), and ranges were calculated for the quantitative characters for each season. In addition, best linear unbiased prediction (BLUP) analysis was performed to model the quantitative trait data over two seasons using the method described by Wen et al.[36] and JMP statistical software (v. 14). BLUPs were used for all further statistical and association analysis of quantitative traits. The percentage distributions for qualitative characters were calculated using descriptive statistics. Broad-sense heritability was calculated according to the formula $H^2 = Vg/Vp \times 100$[37,38] where, Vg (σ^2g) = genotypic variance; Vgxy (σ^2gxy) = genotypic x year interaction variance; Ve (σ^2e) = error variance; and Vp (σ^2p) = phenotypic variance = σ^2g + σ^2gxy/y + σ^2e/ry (r = number of replicates, y = number of years). Variance components (Vg, Vp, and Ve) were calculated with ANOVA analysis (Type III sum of squares). Genotypic coefficient of variation (GCV) and phenotypic coefficient variation (PCV) were calculated using the formulae: GCV % = $\sqrt{Vg}/\bar{x} \times 100$ and PCV % = $\sqrt{Vp}/\bar{x} \times 100$.[39] Spearman's rho correlation coefficient was used to study the relationships between variables. All calculations were done using PAWS statistics software (SPSS Inc. Released 2009, PASW Statistics for Windows, Version 18.0, Chicago).

SSR marker amplification

DNA was extracted from young, ground leaf tissue using a cetyltrimethylammonium bromide (CTAB) method according to Doyle and Doyle.[40] The integrity of DNA was assessed by gel electrophoresis. DNA was quantified by spectrophotometer (Thermo Scientific, Multiskan GO). A total of 100 SSR primer pairs were used for genotyping 101 of the 114 accessions. Thirteen accessions were excluded due to the low quality of them. The SSR marker primer pairs were based on previous work: 42 SSR markers,[41] 26 FbgSSR markers,[18] 13 GBSSR-VF markers,[20] and 19 VfGSSR markers.[13] The GBSSR-VF and VfGSSR primer pairs were amplified as described by Göl et al.[42] Primer sequences and annealing temperatures are in supplementary materials (Table S4). SSR amplification for the FbgSSR and Ma et al.[41] SSR primer pairs were performed with 30 ng DNA in a final volume of 20 µL containing 2 µL 10x PCR buffer, 1 pmol forward and reverse primers, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 U Taq Polymerase. PCR conditions were 95°C for 4 min for one initial denaturation cycle, followed by 35 cycles of 45 s at 95°C for denaturation, 1 min for annealing, and 1 min at 72°C for extension, and the final extension cycle was at 72°C for 5 min. PCR reactions were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems). PCR products were separated using a capillary electrophoresis instrument (Fragment Analyzer Automated CE System; Advanced Analytical) using the DNF-900 dsDNA Reagent Kit (Advanced Analytical), and SSR alleles were visualized with PROSize 3.0 software v. 3.0.1.6 (Advanced Analytical).

Population structure analysis

Allelic data were scored: "1" for presence, "0" for absence, or "9" for missing data to generate binary data. Genetic diversity in the population was calculated using DARwin 6 (Dissimilarity Analysis and Representation for Windows) software[43] with the Dice coefficient. Population structure was determined with Structure 2.2.3 software,[44] which assigned genotypes into subpopulations and produced a Q matrix. Models with 2–10 subpopulations (K = 2–10) were tested for 20 iterations. Burn-in period was 100,000, and the number of Monte Carlo Markov Chain repeats was 100,000. Structure Harvester computer program[45] was used to process results for implementation of the Evanno method[46] to calculate ΔK values for each model based on posterior probabilities. The model with the highest ΔK was selected as the best fit.

Association mapping

The data generated with the SSR primers were used for association mapping of 26 morphological characters. Before conducting association mapping analysis, minor SSR alleles (below 1% frequency) were removed. Four association mapping models were tested: the general linear model (GLM), GLM based on the Q matrix, mixed linear model (MLM), and MLM based on kinship (K) and Q matrix. LD values (r^2 and p-values) between SSR loci were calculated using TASSEL v2.1 software[47] using the rapid permutation test with 10,000 shuffles. SSR loci with p values less than 0.001 were considered to be associated with a given trait. Phenotypic variance explained (PVE) by individual markers (r^2) was used to estimate the QTL effect. QTLs were classified as major and minor based on a threshold PVE value of 10%.[48,49] Q-values were calculated to measure significance in terms of the false discovery rate for the whole set of p-values with a threshold Q value of 0.2.[50]

RESULTS AND DISCUSSION

Morphological trait diversity

The 114 faba bean accessions were assessed for quantitative and qualitative vegetative, inflorescence, pod, and seed traits over two growing seasons (Table S5). Although faba bean is a seed crop, analysis of its vegetative traits is important because traits such as leaflet and stem number help determine photosynthetic capacity that, in turn, affects yield. Floral traits are also significant because they determine earliness and influence pod and seed number.

Quantitative characteristics

Fourteen quantitative traits were examined across two seasons including three vegetative growth traits: numbers of leaflets per leaf and stems per plant and plant height; two inflorescence traits: days

to flowering and number of flowers per inflorescence; six pod traits: pod length, numbers of pods per node and plant, maximum numbers of ovules and seeds per pod, and seed fertilization; and three seed traits: water content of fresh seeds, 100-seed weight, and seed yield. Season effect was statistically significant for most of the quantitative traits ($p < 0.05$, data not shown); therefore, averages and other statistics are given separately for each season (Table 1). Because of the great number of traits, only those which were most variable showed statistically significant differences between landraces and cultivars ($p \leq 0.05$; Table S6) or were studied previously are discussed here.

Leaves of the faba bean accessions had an average of five leaflets with little variability across accessions (Table 1). These results agree with the work of Terzopoulos et al.[51] who examined Greek accessions. However, when measured in the same way, South Tunisian accessions had only 3.5 leaflets per leaf.[52] Duc[1] reported that faba bean leaflet number increased from the bottom to the top of the stem. Because our material was approximately twice as tall as the Tunisian accessions, the difference in leaflet number may result from plant height variability.

The number of stems produced by each plant was quite variable ranging from 1.7 to 14.6 with an average of approximately 5 stems in both seasons (Table 1). Greek and South Tunisian accessions had three to four stems per plant.[51,52] This variation is most likely due to environmental conditions as it is known that stem number has low to moderate broad-sense heritability, which has been estimated as 18%,[53] 32% (this work), and 63%[37] by different studies. The most stems per plant were observed in two Turkish landraces (CGN10382.1 and TR44862) that are naturally adapted to the climate conditions under which the material was grown. These landraces might be useful for breeding this trait that had relatively low but significant positive correlations between seed weight and seed yield ($r = 0.20$ and 0.16; Table S7).

Plants ranged from 38.3 to 180.0 cm tall over the two seasons (Table 1). Plants in season 1 were generally taller than in season 2. The tallest accession was cultivar CGN7730 (Czech Republic; Table S5). Some landraces were also nearly as tall such as CGN07781 (Netherlands) and CGN7843 (Italy). The shortest accessions were NGB8640 (Finland) and TR71255 (Turkey), which were approximately 40% shorter than the overall mean height. Plant height indirectly affects yield. Kumar et al.[54] reported an average height of 117.8 cm for 65 genotypes, which is similar to our result (approximately 106 cm). Al Barri and Shtaya[55] found that Palestinian genotypes had much shorter average height (76.8 cm); however, their material was grown without fertilization or irrigation. Indeed, Della[56] reported significant differences in height for 101 Cypriot accessions when the plants were grown with or without irrigation. Moreover, Hegab et al.[57] found that faba bean height was significantly reduced with delayed sowing. Thus, it is clear that environmental and cultivation factors strongly influence faba bean plant height. This is reflected in the relatively low broad-sense heritability for this trait (28%) in our study.

Days to flowering varied from 53 to 137 days requiring approximately 90 days over all genotypes (Table 1). The cultivars flowered slightly, but not significantly, later than the landraces. NGB1547.1 from Finland was the earliest flowering accession and reached 50% flowering 59 days after seed sowing (Table S5). The other early genotypes were landrace CGN7827.1 (Ethiopia) and cultivar CGN19993 (Netherlands). Early flowering is usually a sought-after trait, however, it can result in frost damage. Herzog[58] reported that European faba bean populations can tolerate chilling from 0°C to 10°C and can even survive brief exposure to temperatures as low as −5°C. Izmir has a

TABLE 1 Quantitative morphological traits for the faba bean materials.

	Season 1			Season 2			
Trait	**Mean ± SE**	**Range**	**CV (%)**	**Mean ± SE**	**Range**	**CV (%)**	**Heritability (%)**
Leaflets per leaf	5.57 ± 0.05	4.00–7.00	10.51	5.33 ± 0.07	2.60–6.60	14.10	55.6
Stems per plant	4.65 ± 0.12	2.20–8.80	27.43	4.94 ± 0.19	1.67–14.60	41.77	32.3
Plant height, cm	120.39 ± 2.49	62.00–180.00	22.09	93.14 ± 2.23	38.33–166.00	25.54	27.5
Days to flowering	99.31 ± 2.77	53.00–137.00	29.77	84.11 ± 1.54	65.00–127.00	19.60	41.2
Flowers per inflorescence	5.15 ± 0.10	2.13–8.00	21.12	4.88 ± 0.12	1.80–8.80	25.59	55.6
Pod length, cm	10.06 ± 0.23	4.40–20.80	24.72	9.3 ± 0.22	5.20–16.12	24.98	75.5
Number of pods per node	1.39 ± 0.04	1.00–2.67	27.09	1.35 ± 0.03	1.00–2.40	25.94	38.4
Number of pods per plant	32.19 ± 2.30	5.50–145.50	74.75	22.72 ± 1.36	2.33–64.80	63.91	88.5
Maximum number of ovules per pod	3.56 ± 0.05	1.80–4.80	14.15	3.54 ± 0.06	2.40–8.20	18.31	66.1
Number of seeds per pod	3.41 ± 0.05	1.40–4.80	16.36	3.33 ± 0.04	2.20–4.40	14.30	55.3
Seeds fertilization, %	95.39 ± 0.61	73.33–105.00	6.86	94.7 ± 0.76	53.66–100.00	8.60	9.6
Water content of fresh seeds, %	76.10 ± 0.48	56.67–84.76	6.59	69.33 ± 0.55	47.36–81.83	8.44	41.1
100-seed weight, g	79.77 ± 3.06	29.73–171.08	40.75	75.72 ± 3.17	22.22–179.60	44.68	88.6
Seed yield, g	57.12 ± 3.86	5.38–280.81	69.83	55.53 ± 3.62	2–167.65	68.01	77.0

Note: Means, ranges, and CVs are given for Season 1 and Season 2 separately. Broad-sense heritability is also included.

moderate climate; the coldest month in the year is January with temperatures between 5°C and 11°C and it rarely snows.[59] However, season 1 was exceptional as the temperature fell below zero (−3°C) and there was snowfall at the beginning of January. The plants survived, however, their flowering was delayed resulting in a statistically significant difference in flowering time over the two seasons and a moderate broad-sense heritability (41%). This result was expected as Açikgöz[60] stated that low temperatures delayed flowering date in the genus *Vicia*.

Average pod length varied from 4.4 to 20.8 cm during the two seasons (around 9.0 cm on average) with the shortest pods in CGN7751 (unknown origin) and CGN13487 (Pakistan; Tables 1 and S5). The longest pods were observed in the Turkish cultivar CGN19987 followed by the Spanish cultivar CGN19979 with around 16.0 cm pods. The cultivars' pods were significantly ($p < 0.005$) longer than landraces (Table S6). Pod length was highly heritable with broad-sense heritability of 75.5%, which agreed with a previous study that estimated an even higher value (98%).[61] There were significant positive correlations between pod length and many characters; 100-seed weight, pod angle, seed coat color, and pod shape ($r = 0.44$ to 0.65, $p < 0.01$) had the closest relationships with this trait (Table S7). Moreover, pod length was negatively correlated with seed shape ($r = -0.57$, $p < 0.01$). Al Barri and Shtaya[55] and Musallam et al.[62] also reported strong correlations between pod length and 100-seed weight ($r = 0.88$ and 0.74, respectively). Therefore, pod length is an important morphological trait associated with yield.

Number of pods per plant was the quantitative trait with the most variation (CV 63.9%–74.8%; Table 1). Plants had from 2.3 to 145.5 pods over the two seasons. Landraces CGN10385 (Turkey) and CGN7719 (Italy) had the fewest pods (Table S5). Notably, CGN7719 also had very few stems perhaps explaining its lack of pod set. Landrace CGN7740 (Egypt) consistently produced more than 60 pods each season suggesting that it could be useful for breeding a new high-yielding cultivar. Pod number per plant had one of the highest broad-sense heritability for the quantitative traits (89%) and was strongly correlated with seed yield ($r = 0.78$, $p < 0.01$; Table S7). In a previous study, strong positive correlations were reported between pod number per plant and seed number per plant (92%, $p < 0.001$), as well as plot yield (67%, $p < 0.001$).[63] These results indicate that direct selection for pod number could be useful for increasing seed yield in breeding programs. However, other studies indicated that the pod set was highly dependent on the environment with very low broad-sense heritability estimates (2%–3%).[53,64] Such contradictions may be encountered when studies are compared especially if growing conditions such as irrigation and fertilization are variable.

Maximum number of ovules per pod ranged from 1.8 to 8.2 with an average of approximately 3.5 ovules over all genotypes in the two seasons (Table 1). A significant difference ($p < 0.005$) was detected between landraces and cultivars with the cultivars producing slightly but significantly more ovules than landraces in both seasons (Table S6). The cultivars CGN19987 (Turkey) and CGN19979 (Spain) had the most ovules with low variation over seasons (Table S5). Both of these accessions had the longest pods in the population. Among the landraces, CGN7757 (Greece) and CGN7719 (Italy) stably produced the most ovules suggesting that they are interesting breeding materials. As expected, this trait was significantly correlated with the number of seeds per pod ($r = 0.39$, $p < 0.01$; Table S7). In our study, approximately 5% of the ovules did not develop into seeds.

Seed number per pod ranged from 1.4 to 4.8 with approximately 3.4 seeds on average (Table 1). Cultivars produced, slightly but significantly, more seeds than landraces in both seasons ($p \leq 0.05$; Table S6). The most seeds were produced by cultivars CGN19979 (Spain) and CGN18862 (Netherlands); however, certain landraces also had high seed numbers: NGB8643 (Finland) and CGN7719 (Italy; Table S5). The fewest seeds were produced by landraces CGN07734 (Ethiopia) and TR44862 (Turkey). The latter accession was also recorded as producing the most stems in this study; therefore, seed production may have been sacrificed for vegetative growth. Seed number per pod was moderately heritable with a broad-sense heritability of 55%, which is consistent with the result of a previous study[63] where it was reported as 62%.

Seed weight was determined for a bulk sample of 100 seeds and showed considerable variability in the accessions (CV 40.8%–44.7%) ranging from 22.2 to 179.6 g (Table 1). According to the classification of Cubero,[65] the majority of genotypes (63.1%) were classified as medium-seeded with 100-seed weight ranging from 51.1 to 95.4 g. Large-seeded types made up 21.9% of genotypes with values from 100.5 to 174.6 g (Figure S1). The third group (14.9%) consisted of small-seeded individuals with values from 29.4 to 48.6 g. Average seed weight of the cultivars was 10% higher than for the landraces indicating the impact of selection and breeding. Thus, as expected the highest 100-seed weights were for cultivars: CGN19985 (unknown origin; approximately 175 g) followed by three Turkish cultivars: Eresen87, Filiz99, and Salkim (approximately 156 g; Table S5). The prevalence of Turkish material in the rankings for seed weight was not unexpected as large seeds are preferred in Turkey.[53,66] Cultivars also had the lowest weights: Mikko from Finland and Kontu from Germany. When grown in Finland, Mikko had even lower seed weight, 22 g versus 29.4 g (our study).[67] Despite its low seed weight and yield, Mikko is cultivated in Finland due to its extremely early maturation.[68] The yield limitations of Mikko were apparent in our work as this cultivar had the fewest flowers per inflorescence. Interestingly, 100-seed weight was negatively correlated with seed shape ($r = -0.69$, $p < 0.01$) indicating that rounder seeds are often smaller than flattened seeds (Table S7). Broad-sense heritability for 100-seed weight was previously reported as 66%[63] and 99%,[37] which is consistent with our work (89%). These results indicate that direct selection based on mean weight would be an effective way to improve this trait.

Dry seed yield was the second most variable quantitative trait in the population (CV 68.0%–69.8%; Table 1). Seed yield varied from 2.0 to 280.8 g across two seasons with an average of around 55.0 g per plant. Cultivar CGN19987 (Turkey) and landrace CGN10371 (Algeria) had the highest yields (around 150.0 g) with little difference between the two seasons suggesting yield stability (Table S5). CGN7719 (Italy), a landrace, and CGN18892 (Netherlands), a cultivar, had the lowest yields with approximately 6.0 g each year. CGN7719 grew poorly under our conditions and was one of the accessions with the fewest stems and pods. This trait was also correlated with the number of

pods per plant ($r = 0.78$, $p < 0.01$; Table S7) as expected because pods per plant is a major yield factor.[69]

Qualitative characteristics

Data for 12 qualitative traits were collected for the 61 landraces and 53 cultivars over 2 years (Table 2). Season effect was not statistically significant, in other words, there was no year effect on the qualitative traits ($p > 0.05$, data not shown). Four vegetative traits were examined: leaflet shape, stipule spot pigmentation, stem pigmentation at flowering, and mature stem color. In addition, flower and seed color were assessed as were seed shape and pod characteristics. None of the parameters showed significantly different distributions of trait categories in cultivars versus landraces. Therefore, only traits that were highly heritable or significantly correlated with other characteristics are described here.

TABLE 2 Qualitative morphological traits for the faba bean materials.

Trait	Class description	Landraces (%)	Cultivars (%)	Landraces and cultivars (%)	Heritability (%)
Leaflet shape	Narrow	14.8	18.9	16.7	95.8
	Intermediate	70.4	66.0	68.4	
	Round	14.8	15.1	14.9	
Stipule spot pigmentation	Absent	0.0	9.4	4.4	54.2
	Present	100.0	90.6	95.6	
Stem pigmentation at flowering	Absent	0.0	7.5	3.5	45.4
	Weak	26.2	21.2	23.9	
	Intermediate	49.2	46.2	47.8	
	Strong	24.6	25.0	24.8	
Mature stem color	Light	18.0	15.1	16.7	100.0
	Dark	82.0	84.9	83.3	
Intensity of petal streaks	No streaks	0.0	7.7	3.5	64.4
	Slight	27.9	13.5	21.2	
	Moderate	41.0	42.3	41.6	
	Intense	31.1	36.5	33.6	
Wing petal color	Uniformly White	0.0	9.4	4.4	37.9
	Spotted	100.0	90.6	95.6	
Pod angle	Erect	85.2	67.9	77.2	100.0
	Horizontal	13.1	22.6	17.5	
	Pendent	1.6	9.4	5.3	
Pod shape	Subcylindrical	68.9	67.9	67.5	96.3
	Flattened	31.1	32.1	32.5	
Pod surface reflectance	Matte	27.9	26.4	27.2	95.6
	Glossy	72.1	73.6	72.8	
Seed shape	Flattened	50.8	50.9	50.9	93.1
	Round	49.2	49.1	49.1	
Seed coat color	Black	6.6	1.9	4.6	53.5
	Dark brown	3.3	3.8	3.7	
	Light brown	52.4	52.8	53.7	
	Light green	23.0	26.4	25.9	
	Dark green	1.6	0.0	0.9	
	Violet	6.6	1.9	4.6	
	White	0.0	9.4	6.5	
	Mixed	6.6	3.8	5.3	
Hilum color	Black	86.9	75.5	81.6	86.6
	Colorless	4.9	9.4	7.0	
	Mixed	8.2	15.1	11.4	

Note: Percentages of each trait class are given for landraces and cultivars separately and combined. Broad-sense heritability is also included.

Leaflet shape was intermediate for most landraces and cultivars and this trait had very high broad-sense heritability, 96% (Table 2). In soybean, plants with intermediate leaflets had more pods per plant and higher yield than those with either ovate or narrow leaflets.[70,71] However, we did not observe similar correlations in faba bean (Table S7).

All landraces had brown spots on the stipule, while 9.4% of the cultivars lacked this brown pigment (Table 2). Stipule spot pigmentation was significantly correlated with wing petal color ($r = 1.0$, $p < 0.01$) and also seed hilum color ($r = -0.78$, $p < 0.01$) such that plants with stipule pigmentation always had spotted wing petals and usually had black hila (Table S7).

Mature stem color was similar for both landraces and cultivars with the majority of genotypes having dark color (Table 2). This trait was completely controlled by genetic factors with a heritability of 100%. Another important trait is flower color. This characteristic can be used as a morphological marker for seed tannin content because anthocyanins and tannins are derived from the same precursors.[72] Mutation in this pathway results in faba bean with no pigmentation in stems and stipules, white flowers, and no or low tannin in seeds. Therefore, as expected, the genotypes that had these flower characters in our study also had low seed tannins (unpublished data).

The majority of plants had erect pods: 85.2% and 67.9% for landraces and cultivars, respectively (Table 2). Horizontal pods were observed in 13.1% of landraces and 22.6% of cultivars. Only a few landraces and cultivars had pendent pods. Pod angle was highly heritable (100%) and significantly and moderately correlated with 100-seed weight ($r = 0.49$, $p < 0.01$) and pod length ($r = 0.63$, $p < 0.01$; Table S7). Therefore, more horizontal and pendent pods tended to be longer and have larger seeds.

Seed shape of the landraces and cultivars was equally divided between flattened and round (Table 2). This trait was highly heritable with broad-sense heritability of 93%. All of the landraces and cultivars with 100-seed weight above 88.8 g had flattened shape (Table S5). Seed shape was negatively correlated with pod length ($r = -0.57$, $p < 0.01$), angle ($r = -0.49$, $p < 0.01$), and seed coat color ($r = -0.40$, $p < 0.01$) such that flat seeds were more likely to be lighter in color and found in shorter, pendent pods (Table S7).

There was high diversity among faba bean genotypes for seed coat color (Table 2 and Figure S2). The predominant color for landraces and cultivars was light brown (52%), followed by light green (approximately 24%) with white seeds only seen in cultivars. Indeed, white was the third most common seed color in cultivars (9.4%). Landraces had equal proportions of black, violet, and mixed seeds (6.6%). Coat color was moderately heritable (54%) and positively correlated with 100-seed weight, pod length, and angle as previously mentioned ($r = 0.32$–0.45, $p < 0.01$; Table S7). Coat color was also correlated with stipule spot pigmentation and wing petal color ($r = 0.39$, $p < 0.01$) indicating that plants with colored stipules and petals tend to have darker colored seeds.

The majority of landraces and cultivars had black hila (Table 2 and Figure S2). Seeds with colorless hila were seen in both landraces (4.9%) and cultivars (9.4%). All white seeds had colorless hila and white wing petals. This relationship was also reflected in the correlations between the color traits. Unlike most of the other color traits, hilum color had very high broad-sense heritability of 87%.

Association mapping

The 100 SSR primer pairs generated 651 polymorphic fragments. The genetic diversity within the population was calculated using the Dice coefficient. The mean pairwise genetic diversity was 0.34 and ranged from 0.20 to 0.49, which indicates sufficient variation for association analysis (data not shown). Analysis revealed that the population structure was best represented as two subclusters (the highest ΔK at $K = 2$; Figure S3). Therefore, the Q matrix for $K = 2$ was used for QTL detection. The GLM had the highest proportion of significant results among the four association models (12%) and was used for association mapping (as determined from $\pi 1$; Table S8). LD analysis generated a total of 211,575 pairwise comparisons of 651 SSR loci across 114 genotypes. Overall, 1% of marker pairs had significant LD levels ($p \leq 0.01$ and $r^2 \geq 0.01$). PVE values of individual alleles (r^2) ranged from 10% to 22% and all were classified as major effect loci (PVE > 10%; Table 3). While the inability to detect loci with small effect is often cited as a limitation of association mapping, such loci would be of questionable usefulness in breeding faba bean. Only markers with p-values ≤0.001 and Q-values ≤0.20 were considered to be significant. As a result, association mapping identified 75 significant SSR marker–trait associations for 20 of the 26 characters (Table 3). No SSR marker alleles were identified for leaflets per leaf, days to flowering, number of pods per plant, maximum number of ovules per pod, leaflet shape, and pod angle. Only marker associations with the highest PVEs are highlighted below.

Eighteen QTLs were associated with five of the seven vegetative traits (Table 3). The highest effects were seen for SSR7150-106, which was linked to stems per plant (21%) and both VfG4-285 and VfG27-298, which were linked to stipule spot pigmentation (19%). Indeed, SSR7150-106 and stems per plant were the second most significant marker–trait association in this study ($p = 3.6 \times 10^{-6}$). The rest of the marker alleles for the vegetative traits accounted for 10%–16% of variation (approximately 13%).

Eleven SSR marker alleles were associated with three of the four inflorescence traits (Table 3). Wing petal color had the most significant marker–trait associations with VfG4-285 ($p = 5.2 \times 10^{-6}$, PVE = 19%) and VfG27-298 ($p = 8.2 \times 10^{-6}$, PVE = 19%). The remaining markers' PVE values ranged from 10% to 14% for the inflorescence traits.

Thirteen QTLs were detected for six of the nine pod traits (Table 3). The most significant marker–pod trait association was VfG9-124 ($p = 5.1 \times 10^{-6}$), which was linked to pod surface reflectance with the highest PVE value (19%) among the pod traits. VF22-266 and VfG1-263 were significantly associated with pod length ($p = 8.7 \times 10^{-5}$) and seed fertilization ($p = 4.3 \times 10^{-5}$) accounting for 15% and 16% of the variance for the traits, respectively. The rest of the markers had moderate PVE values ranged from 10% to 14% with an average of 13%.

TABLE 3 Faba bean simple sequence repeat (SSR) markers associated with morphological traits as identified with general linear model (GLM).

Trait	Locus	PVE* (%) (r^2)	*p-value* ≤0.001	Q-value ≤0.2
Vegetative				
Stems per plant	SSR7150-106	21	3.6E−06	1.7E−03
	SSR7150-100	11	9.1E−04	1.3E−01
	VfG87-323	10	1.1E−03	1.3E−01
	FbgSSR643-230	11	1.1E−03	1.3E−01
Plant height	VfG87-183	13	2.2E−04	1.2E−01
Stipule spot pigmentation	VfG4-285	19	5.2E−06	1.5E−03
	VfG27-298	19	8.2E−06	1.5E−03
	VfG34-221	11	8.5E−04	8.6E−02
	SSR5204-245	10	1.1E−03	8.6E−02
	FbgSSR451-295	11	1.2E−03	8.6E−02
Stem pigmentation at flowering	VfG53-263	13	3.3E−04	1.8E−01
Mature stem color	VfG53-126	16	5.7E−05	1.4E−02
	VfG53-278	15	1.0E−04	1.4E−02
	VF8-292	14	1.4E−04	1.4E−02
	VfG53-323	15	1.6E−04	1.4E−02
	VfG47-327	14	1.8E−04	1.4E−02
	VfG1-206	11	1.0E−03	6.2E−02
	VfG13-203	11	1.2E−03	6.3E−02
Inflorescence				
Flowers per inflorescence	SSR2833-220	13	3.3E−04	1.1E−01
	SSR745-245	11	6.1E−04	1.1E−01
Intensity of petal streaks	FbgSSR30-118	14	1.7E−04	9.5E−02
	FbgSSR322-234	11	6.4E−04	1.4E−01
	VF153-245	11	7.6E−04	1.4E−01
	FbgSSR229-210	12	9.6E−04	1.4E−01
Wing petal color	VfG4-285	19	5.2E−06	1.5E−03
	VfG27-298	19	8.2E−06	1.5E−03
	VfG34-221	11	8.5E−04	8.6E−02
	SSR5204-245	10	1.1E−03	8.6E−02
	FbgSSR451-295	11	1.2E−03	8.6E−02
Pod				
Pod length	VF22-266	15	8.7E−05	4.2E−02
	S282-354	14	2.0E−04	4.8E−02
	FbgSSR382-288	10	1.2E−03	1.9E−01
Number of pods per node	VfG47-327	13	2.2E−04	6.3E−02
	VfG27-195	13	3.0E−04	6.3E−02
	VfG87-230	12	3.3E−04	6.3E−02
Number of seeds per pod	VfG10-196	13	2.7E−04	1.6E−01
Pod shape	FbgSSR309-306	14	1.2E−04	5.7E−02
	VfG67-102	12	4.1E−04	8.1E−02
	VfG4-277	12	5.3E−04	8.1E−02
Pod surface reflectance	VfG9-124	19	5.1E−06	2.6E−03
Seed fertilization	VfG1-263	16	4.3E−05	2.2E−02
	FbgSSR675-262	15	1.7E−04	4.3E−02

(Continues)

TABLE 3 (Continued)

Trait	Locus	PVE* (%) (r^2)	*p-value* ≤0.001	Q-value ≤0.2
Seed				
Water content of fresh seeds	VfG47-218	13	3.4E−04	1.9E−01
	SSR2615-176	11	6.7E−04	1.9E−01
100-seed weight	S282-354	22	2.7E−06	1.2E−03
	SSR8308-230	19	6.1E−06	1.3E−03
	VF20-196	14	1.7E−04	2.4E−02
	SSR7787-285	13	2.7E−04	2.9E−02
	SSR8308-250	12	3.7E−04	3.2E−02
	SSR5366-125	12	5.3E−04	3.8E−02
	SSR1932-235	14	6.1E−04	3.8E−02
	VF131-220	11	7.2E−04	3.9E−02
	SSR1788-155	11	9.5E−04	4.6E−02
	VF20-276	11	1.2E−03	4.7E−02
	VfG1-242	11	1.2E−03	4.7E−02
Seed yield	VfG31-211	13	1.8E−04	7.9E−02
	FbgSSR564-265	12	4.8E−04	7.9E−02
	VF19-196	11	6.9E−04	7.9E−02
	VfG31-341	11	7.5E−04	7.9E−02
	VF52-257	11	8.5E−04	7.9E−02
	FbgSSR293-242	11	9.4E−04	7.9E−02
	VfG1-335	11	1.1E−03	7.9E−02
	VfG3-155	10	1.1E−03	7.9E−02
Seed shape	FbgSSR675-254	13	6.3E−04	1.3E−01
	VF19-203	11	7.6E−04	1.3E−01
	SSR7097-385	10	1.2E−03	1.3E−01
	SSR1932-235	12	1.3E−03	1.3E−01
Seed coat color	FbgSSR451-295	19	2.2E−05	1.2E−02
Hilum color	SSR6198-410	18	2.6E−05	8.5E−03
	VfG4-285	13	4.0E−04	5.6E−02
	VfG27-298	13	5.6E−04	5.6E−02
	FbgSSR293-276	12	7.2E−04	5.6E−02
	FbgSSR545-213	12	9.2E−04	5.6E−02
	VfG34-221	12	1.0E−03	5.6E−02
	FbgSSR451-295	11	1.3E−03	6.2E−02

*PVE indicates the phenotypic variation explained by individual markers.

Twenty-five different SSR markers with 33 associations were linked to the six seed traits (Table 3). The most significant marker–trait associations were S282-354 ($p = 2.7 \times 10^{-6}$, PVE = 22%) and SSR8308-230 ($p = 6.1 \times 10^{-6}$, PVE = 19%), which both were linked to 100-seed weight. Following these, FbgSSR451-295 was linked to seed coat color ($p = 2.2 \times 10^{-5}$ PVE = 19%), and SSR6198-410 was linked to hilum color ($p = 2.6 \times 10^{-5}$ PVE = 18%).

There are very few association mapping studies for yield traits in faba bean. Sallam et al.[28] used 156 SNP markers to scan a population composed of 189 single seed descent lines for determination of marker–trait associations. They detected 12, 9, 2, and 1 QTL for seed yield, days to flowering, plant height, and 100-seed weight, respectively, with PVE values ranging from 3% to 9%.[28] Ávila et al.[73] used a genetic map consisting of 449 markers [RAPD, EST, resistance gene analog (RGA), SSR, isozymes, and gene-derived markers] in an *equina* × *paucijuga* RIL population of 124 individuals. In this work, they revealed a total of 49 marker–trait associations for plant architecture and yield traits. Aguilar-Benitez et al.[32] used a population of 124 RIL individuals derived from a cross of Vf6 (*equina*) × Vf27 (*paucijuga*) parents to conduct QTL analysis for flowering time traits. They

incorporated 26 polymorphic molecular markers, which were derived from candidate genes, into the previously developed maps containing RAPD, EST, and SNP markers. As a result, they detected 12 significant QTLs (PVE 5.3%–17.9%) for flowering time. In another study, Puspitasari[74] performed association analysis using 1322 polymorphic markers (AFLP and SNP) in 189 inbred lines derived from the Göttingen Winter Bean Population and identified five significant marker–trait associations for first flower position, flowering time, plant height, seed yield, and thousand kernel weight (PVE 7%–10%).

Recently, the increased availability of next-generation sequencing platforms has facilitated faba bean QTL studies. In their study, Zhao et al.[75] constructed the first ultradense genetic map comprising 12,023 SNP markers, spanning 1182.65 cM, using the faba bean 130 K targeted next-generation sequencing (TNGS) genotyping platform in an F2 population of 121 plants. In the same study, they identified 65 QTLs associated with 100-seed weight, seed shape, seed coat color, and nutritional quality.[75] Li et al.[33] constructed two high-density genetic maps for two F2 populations derived from three purified faba bean lines using a TNGS genotyping platform. They detected 98 significant QTLs (PVE 1.6%–24.9%) associated with flower, pod, plant type, and grain-related traits.[33] Karaköy et al.[76] performed a genome wide association study (GWAS) in 372 Turkish genotypes using 23,661 diversity array technology sequence (DArTseq) markers and genotyping by sequencing (GBS) analysis. They identified 34 DArTseq marker–trait associations for plant structure and reproductive traits.[76] Recently, Skovbjerg et al.[77] genotyped 2678 faba bean accessions using the Vfaba_v2 Axiom SNP array, which includes around 60 K probes. In this study, they conducted GWAS analysis with 21,345 SNP markers, which resulted in the identification of 238 significant marker–trait associations for growth, reproductive, and seed-related traits.[77] Because similar markers were not used in the aforementioned studies, we cannot compare the locations of their loci with ours.

Marker co-localizations

Co-localization of marker alleles for different traits indicates the presence of linked genes or pleiotropy, a single gene influencing multiple traits. Eight SSR alleles were associated with more than one trait. QTLs linked to VfG4-285, VfG27-298, VfG34-221, SSR5204-245, and FbgSSR451-295 were detected for both stipule spot pigmentation and wing petal color. Among these, both VfG4-285 and VfG34-221 were also linked to hilum color, and FbgSSR451-295 was also linked to both hilum color and seed coat color. Because all of these traits are related to organ color, it is likely that these are examples of pleiotropy. In fact, the pleiotropic association between stipule spot and flower color has been known for a long time.[78] Stipule spot pigmentation is used as a morphological marker as its presence or absence indicates flower color and tannin content.[79] In our study, as expected, a perfect positive correlation (100%) was detected between stipule spot pigmentation and wing petal color. Zero-tannin traits, such as white flower, were reported to be controlled by two genes, zt1 and zt2,[78,80] with pleiotropic effects on seed coat color in faba bean. Later, Khazaei et al.[79] found that stipule spot pigmentation was under the control of two unlinked genes, spp1 and spp2, and proposed that the latter gene is the same as zt2 (controlling white flower). Khazaei et al.[79] also confirmed the observations of Rowlands and Corner[81] that the pleiotropy of stipule spot pigmentation and flower color did not include hilum color. However, in our study, VfG4-285, VfG34-221, and FbgSSR451-295 were significantly associated with hilum color as well as stipule spot pigmentation and wing petal color. This was also observed by Metz et al.[82] who reported linkage between all zero-tannin traits, such as colorless hilum, colorless stipule spot, and white flower in faba bean. Clearly, the genetic control of organ color traits in faba bean merits further study.

SSR allele S282-354 was associated with QTLs for pod length and 100-seed weight. This result and the positive correlation between these two traits ($r = 0.65$, $p < 0.01$) indicate that plants that produced longer pods had larger seeds. Two traits, 100-seed weight and seed shape, were associated with SSR1932-235 and were negatively correlated ($r = -0.70$; $p < 0.01$) in this study. This result suggests that the use of flattened seed shape as a morphological indicator of heavier seeds has a genetic basis. In agreement with this, a previous study by Zhao et al.[75] discovered two QTLs significantly linked to 100-seed weight and seed morphology-related traits (seed area, seed perimeter, seed length, and seed width). Additionally, the study revealed significant positive correlations between 100-seed weight and all seed morphology-related traits. These results suggest a potential shared genetic basis for these traits.

CONCLUSION

The primary goal in faba bean breeding is the improvement of yield-related traits and yield stability. For this purpose, conventional breeding programs are based on the selection of individual plants using phenotype. Therefore, it is very important to characterize germplasm for agro-morphological traits. In this study, 114 faba bean landraces and cultivars were characterized for 26 traits providing data that can be used for selection of potential new parents and cultivars.

Historically, breeding studies for yield stability have not been very successful due to the low or moderate heritability of such traits. Moreover, phenotypic mass selection has often been inefficient when applied to faba bean using only yield traits.[83,84] Since it is reported that a single selection of such components resulted in low yield stability, combinations of morphological characters that are indirectly related to yield should be included in breeding programs to maximize the success of yield improvement.[84,85] The shortcomings of phenotypic selection also indicate that molecular marker approaches should be integrated into breeding for yield and yield components. The work presented herein is one of the largest association mapping studies performed in faba bean to date. Based on our results, all significant QTLs had major effects on the traits. Of the identified loci, those for seed weight (S282-354, 22%) and number of stems (SSR7150, 21%) could be the target of MAS for increased yield. In addition, two of the identified seed coat (FbgSSR451-295, 19%) and hilum (SSR6198-410, 18%) color QTLs are of interest for breeding low tannin faba beans.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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