

Article

Genetic Analysis of Main Agronomic Traits and QTL Mapping of Leaf Type in Spinach

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Highlights

We analyzed genetic models for spinach leaf length, width and petiole length using six-generation populations. Leaf-type QTLs were mapped based on a high-density genetic map. We identified *SpPINL2* for leaf width and *SpFAR1-ULP-zf-GRF* for petiole length.

What are the main findings?

- The optimal genetic models under different cultivation patterns were MX1-AD-ADI for leaf length and leaf width, and MX2-ADI-AD for petiole length.
- *SpPINL2* and *SpFAR1-ULP-zf-GRF* may regulate leaf width and petiole length in spinach, respectively.

What are the implications of the main findings?

- This study clarifies the genetic rules of spinach leaf-related traits under varied cultivation conditions.
- The obtained QTLs and candidate genes facilitate marker-assisted selection and new variety breeding in spinach.

Abstract

Spinach (*Spinacia oleracea* L.) is an important leafy vegetable crop, and its leaf characteristics are important for spinach germplasm innovation and new variety breeding. In this study, spinach inbred lines P₁ and P₂ were used as parents to construct six-generation segregating populations. Based on multi-location trials, we adopted an improved analytical procedure for the major gene plus polygene mixed genetic model, and the genetic models governing leaf length, leaf width and petiole length in spinach were screened. Furthermore, a high-density genetic linkage map of spinach was constructed using re-sequencing data from 152 individuals of the F₂ population, and QTLs (quantitative trait loci) governing spinach leaf type were mapped to identify their candidate genes. The results showed that the optimal genetic model for leaf length was MX1-AD-ADI, for leaf width was MX1-AD-ADI, and for petiole length was MX2-ADI-AD. The genetic map developed from the F₂ segregating population contained 5900 bin markers assigned to six linkage groups, spanning a total genetic distance of 843.48 cM. One QTL associated with leaf length was mapped on chromosome 2 and designated qLL2-1, yet no candidate genes were identified within its candidate interval. Two QTLs controlling leaf width were mapped on chromosome 1 and chromosome 2, designated qLW1-1 and qLW2-1, respectively, and 15 genes were annotated in this candidate region. One QTL related to petiole length was located on chromosome 2 and named qPL2-1, with four genes annotated in its candidate interval. Combined with



Academic Editors: Bin Dong, Guirong Qiao and Shiwei Zhong

Received: 31 May 2026

Revised: 2 July 2026

Accepted: 13 July 2026

Published: 17 July 2026

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spinach genome annotation and expression-level analysis of candidate genes, the gene *SOV2g023140* was identified as the candidate gene regulating spinach leaf width, which was named *SpPINL2*. Meanwhile, three genes regulating petiole length constituted a gene cluster *FAR1-ULP-zf-GRF*, designated *SpFAR1-ULP-zf-GRF*.

Keywords: spinach; QTL; leaf length; leaf width; petiole length

1. Introduction

Spinach (*Spinacia oleracea* L.) belongs to the genus *Spinacia* within the subfamily Amarantoideae of the family Amaranthaceae. It is an annual or biennial herbaceous plant whose primary edible organ is its green leaves [1]. Currently, spinach is cultivated in more than 60 countries, with the global planting area showing an annual upward trend. According to the Food and Agriculture Organization of the United Nations, more than 735 thousand ha of spinach were planted worldwide in 2023 [2]. During the vegetative growth stage of spinach, leaves cluster on the shortened stem. Most leaves are round, elliptic or round-pointed, while a few are halberd-shaped or with several serrated lobes. The leaf color is mostly dark green or green, and a few are red. In the reproductive growth stage, most leaves are halberd-shaped or triangular-ovate and grow on the flower stem [3,4]. The leaf traits of spinach are important traits that affect the marketability of spinach, and are also important traits that are concerned with germplasm innovation and new variety breeding of spinach.

Leaf traits are quantitative traits and susceptible to environmental influences, which constitutes one major challenge in basic and applied research on spinach. Exploring the genetic patterns governing spinach leaf traits via genetic model analysis is of great significance for spinach germplasm innovation and new variety breeding. The analytical procedure for major gene–polygene mixed genetic model identification of quantitative traits based on backcross populations can effectively detect major genes and polygenes underlying plant quantitative traits [5,6]. This model has been applied to *Lagerstroemia indica*, rape, corn, tomato, eggplant, chrysanthemum [7–12], and other plant species. Liu et al. identified 13 QTLs associated with spinach leaf traits using SLAF-seq and KASP genotyping, and nominated *Spo10792*, *Spo21018* and *Spo21019* as candidate genes regulating leaf width [13]. Using GBS-derived SNPs, Ma et al. determined five SNP markers associated with leaf surface texture, 14 markers linked to petiole color, and seven markers correlated with leaf margin traits in spinach [14]. Liu et al. detected a significant correlation between root architecture and leaf traits in spinach, and revealed that genotypes with halberd-shaped leaves tended to develop larger root systems than those with nearly round leaves [15]. Cai et al. mapped five QTLs for leaf color via GBS-based genetic mapping, with *ORF11*, *ORF12* and *ORF17* proposed as candidate genes controlling spinach leaf pigmentation [16].

The polar localization (asymmetric distribution) of *PIN* proteins in plants is highly correlated with the auxin transport direction. *PIN* proteins are considered the primary transporters mediating the asymmetric distribution of auxin. The function of *PIN* proteins is regulated at multiple levels, including transcriptional regulation, subcellular trafficking, and post-translational modification [17]. *PIN* proteins are modulated by various phytohormones and environmental stimuli, including cytokinin, gibberellin, and abscisic acid. The distribution of auxin is regulated by the distribution of *PIN* proteins, which further governs plant growth and development [18]. Eight *PIN* family members designated *PIN1*–*PIN8* have been identified in *Arabidopsis thaliana*. *PIN1* is broadly expressed in multiple organs, localized in the stele and inner cortex of root tips as well as shoot apical meristems; it

participates in gravitropism, embryogenesis, vascular differentiation and leaf primordia initiation [19]. *PIN2* is mainly specifically expressed in the meristem and elongation zone of root tips as well as root caps of lateral roots. Its core function is to mediate basipetal auxin transport in root meristems and elongation zones. Loss-of-function mutants exhibit obvious phenotypes including shortened root length and impaired root gravitropic response. Overexpression of *PIN2* can markedly enhance auxin transport from shoots to roots, and it is a key member in the regulation of root gravity [20]. *PIN3* is highly expressed in vascular tissue and root caps. Upon gravity stimulation, *PIN3* undergoes polar redistribution to mediate root gravitropism; it is also involved in lateral root formation, phototropism and shade avoidance responses [21,22]. *PIN4* is abundantly expressed in the quiescent center of root tips, regulating radicle formation and apical meristem development and participating in phototropic responses [23]. *PIN5* localizes to the endoplasmic reticulum and maintains auxin homeostasis between the cytoplasm and endoplasmic reticulum, contributing to embryogenesis, root elongation and lateral root formation, etc. [24]. *PIN6* is localized at both the endoplasmic reticulum and plasma membrane, and functions in lateral root development and nectar secretion [25,26]. *PIN7* accumulates in apical columella cells and participates in root gravitropism and embryonic polarity establishment [27]. *PIN8* is localized to the endoplasmic reticulum, antagonizes *PIN5* activity, takes part in pollen development, and may modulate the later stages of lateral root initiation [28].

Gene clusters are groups of genes that are physically closely adjacent, functionally related or homologous on chromosomes. Their key features are that they generally participate in the same metabolic pathway or biological process, and jointly regulate secondary metabolite synthesis, plant growth and development, as well as stress defense responses, ensuring the integrity and coordination of biological functions [29,30]. Genes within a gene cluster contain conserved cis-acting elements and regulatory patterns, and exhibit synergistic expression upon induction by specific developmental stages or environmental conditions [31]. The compact linkage distribution and common regulation mode of gene clusters can ensure the integrity of the biological genetic recombination process, and improve the signal transduction and metabolic efficiency of organisms, which is of great significance for organisms to adapt to the complex living environment. The “*FAR1-ULP-zf-GRF*” gene cluster is a local gene cluster formed by physically close neighbors of three functional related genes, *FAR1*, *ULP*, and *zf-GRF*, on the chromosome in the plant genome. It is common in *Arabidopsis thaliana*, rice, and corn, and belongs to the functional coordination type gene cluster [32–34]. *FAR1* and its homolog *FHY3* are key components of the phytochrome A-mediated far-red light signaling pathway [35,36]. Members of the *FAR1* family (*FRS* family) exert specific functions in multiple growth and developmental processes, including the regulation of flowering time, maintenance of meristem activity, and regulation of carbon metabolism during vegetative growth such as hypocotyl elongation. Their transposase-derived structural characteristics are closely associated with functional differentiation of this gene family [37]. *ULP*-mediated *SUMO* modification can also indirectly regulate the growth rate of plant vegetative organs by modulating the activity of growth-related transcription factors, serving as an indispensable regulatory link during plant organ development [38]. *GRF* proteins contain highly conserved domains, which have been identified in *Arabidopsis thaliana*, *Zanthoxylum bungeanum*, corn, wheat, and rice. In *Arabidopsis thaliana*, *GRF/zf-GRF* proteins interact with *ZF-HD* transcription factors; *GRF3* directly activates the expression of *HB33* to regulate leaf size, cell proliferation, and leaf senescence [39].

In this study, spinach inbred lines P_1 and P_2 were used as parents to develop a six-generation segregating population. Genetic analysis of leaf length, leaf width and petiole length in spinach was performed via the mixed major gene plus polygene inheritance model.

Whole-genome resequencing was conducted on the two parents and 152 F₂ individuals for QTL mapping of leaf-related traits, followed by screening of underlying candidate genes. The QTL identification for leaf length, leaf width and petiole length is expected to develop tightly linked molecular markers for marker-assisted breeding and germplasm innovation in spinach.

2. Materials and Methods

2.1. Test Materials

Spinach inbred lines P₁ (code: C₁) and P₂ (code: C₃), provided by the Spinach Research Group of the Vegetable Research Institute, Beijing Academy of Agriculture and Forestry Sciences, were used as parents. The F₁ generation was obtained by crossing P₁ and P₂. The F₂ population was generated by self-pollinating the F₁ plants. Meanwhile, the BC₁ and BC₂ populations were developed by backcrossing the F₁ progeny with parents P₁ and P₂, respectively. Thus, a six-generation genetic population consisting of P₁, P₂, F₁, F₂, BC₁, and BC₂ was constructed.

2.2. Determination of Leaf Type Traits of Spinach

This experiment was a multi-site trial with different cultivation conditions and substrates. Plants in the greenhouse and plastic tunnel were grown in native soil, while open-field cultivation was performed using nutrient pots with dimensions of 5 cm × 7.5 cm × 7 cm filled with commercial seedling substrate for flowers and vegetables, which were placed under open-air conditions. All trials were conducted at the Beijing Experimental Farm, Institute of Vegetables, Beijing Academy of Agriculture and Forestry Sciences. During the experimental period, the average daytime temperature was 24 °C, the average nighttime temperature was 13 °C, the daily average light duration was 8.5 h, and the average relative humidity was 50%. Leaf length, leaf width and petiole length were measured across the six-generation genetic population. Measurements were carried out when spinach developed to the six-leaf vegetative stage using fully expanded functional leaves. Three leaves were sampled and measured per plant, and the average value was taken as the phenotypic datum for each individual plant.

2.3. Genetic Analysis of Leaf Type Traits of Spinach

Using the mixed major gene plus polygene genetic model for plant quantitative traits, multi-generational joint analyses of leaf length, leaf width and petiole length were performed on six-generation spinach populations with SEA2.0 software based on R language 4.3.3 [40]. The maximum likelihood method and IECM algorithm were adopted to calculate the Maximum Likelihood Value (MLV) and Akaike Information Criterion (AIC) for 24 genetic models. All candidate models were subjected to fitness validation, including homogeneity tests (U_1^2 , U_2^2 , U_3^2), the Smirnov test (${}_nW^2$), and the Kolmogorov test (D_n), to screen for the optimal genetic model. The model with the fewest significant statistical indices ($p < 0.05$) was defined as the optimal genetic model. If no significant differences were observed among all candidate models, the model with the lowest Akaike Information Criterion (AIC) value was selected as the final optimal model [41,42]. The least squares method was adopted to estimate the first-order and second-order genetic parameters of the selected model, and to evaluate the genetic effects and heritability of major genes and polygenes.

2.4. QTL Analysis and Expression Analysis of Candidate Genes

Whole-genome resequencing was performed by Beijing Biomarker Technology Co., Ltd. (Beijing, China) (<http://www.biomarker.com.cn/>). A high-density bin-based genetic map was constructed using the bin-mapping strategy.

High-density SNP markers were developed from 152 F₂ spinach individuals via resequencing and HighMap software 1.0 (Beijing Biomarker Technology Co., Ltd. Beijing, China). A bin map was generated for genetic linkage construction and QTL mapping of leaf-related traits to identify trait-linked molecular markers. QTL nomenclature rules: each QTL is named with a lowercase letter q, followed by the abbreviation of the target trait and the corresponding chromosome number. If multiple QTLs for the same trait are detected on a single chromosome, a serial number is appended at the end. For instance, qLW1-1 represents the first QTL associated with leaf width on chromosome 1 in spinach.

2.5. qRT-PCR

The total reaction volume of the qRT-PCR system was 10 µL, containing 1 µL diluted cDNA template, 0.5 µL mixed forward and reverse primers, 5 µL 2 × Taq Pro Universal SYBR qPCR Master Mix (Nanjing Vazyme Biotech Co., Ltd., Nanjing, Jiangsu, China) and 3 µL RNase-free ddH₂O. Reaction procedures were set as follows: initial denaturation at 95 °C for 30 s, followed by 40 amplification cycles. Each cycle consisted of denaturation at 95 °C for 10 s and annealing plus extension at 56 °C for 20 s. A melting curve program was subsequently performed with sequential temperature treatments: 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The 2^{−ΔΔCt} method was adopted to calculate relative gene expression levels. Ct values were calculated as the average of five biological replicates, with five independent technical replicates arranged for each sample. Quantitative amplification was conducted on a Roche LightCycler 480 II instrument (Roche Diagnostics Products (Shanghai) Co., Ltd., Shanghai, China). The housekeeping gene *GAPDH* was used as the internal reference for normalization, and all primer sequences are listed in Table 1. Raw data were sorted and analyzed using Microsoft Excel and GraphPad Prism 8 software.

Table 1. Primers used for qRT-PCR expression analysis.

Gene	Forward Primer	Reverse Primer
SOV1g017280	GAGAAGAAGCTTTGACTTGGCTTACC	CATCCCATTCGTCGTCATAAATGTT
SOV2g023090	CACTCATTATCAGCCGTCTTCC	ATGGTCTTGCCCCACTCTTG
SOV2g023140	TGGCGTGGACAAAGAAACTG	AGGAGCAGCAAAGCATGTGA
SOV2g023050	TTCCTGCAAGAAAGAAACCA	TAGCAATGCTCCTAGACCAATA
SOV2g023130	GCATCGTCTGTTCCGGTGTG	CTTCGTAGAGGAGGAGGTATCA
SOV2g023080	CAAAGAAAGTTCAGCCCCCTAC	ATCCCTATCCGAAAACCTCA
SOV2g003700	GACTTCCTGCCACTTATTCTCA	CACTCATTTTCTCCGACTTCTT
SOV2g003710	CGCTGATAGGTTAACTGCC	TGTGCCAACTTTGAATCTCGT
SOV2g003720	TGATCTCCTGGGTTGTTTCC	CAATCCTTTTACGGGGCTCT
<i>SpGAPDH</i>	CCTGGAGCTGGAAACACAT	TGTGAGAAGCATCCAACAGC

3. Results

3.1. Leaf Phenotypic Characteristics of P₁, P₂, F₁ and F₂ Populations

Phenotypes of both parents and the F₁ generation are presented in Figure 1. Under the three cultivation modes, the average leaf length of parent P₁ ranged from 4.12 to 5.58 cm, the average leaf width ranged from 3.16 to 4.78 cm, and the average petiole length ranged from 0.98 to 2.23 cm. For parent P₂, the average leaf length ranged from 9.64 to 9.83 cm, the average leaf width ranged from 5.33 to 6.28 cm, and the average petiole length ranged from 4.68 to 7.35 cm. For the F₁ generation, the average leaf length ranged from 7.00 to 10.60 cm,

the average leaf width ranged from 4.80 to 6.04 cm, and the average petiole length ranged from 2.58 to 7.08 cm. Petiole lengths of F₁ plants showed significant differences among the three cultivation modes. Detailed data are listed in Table 2.

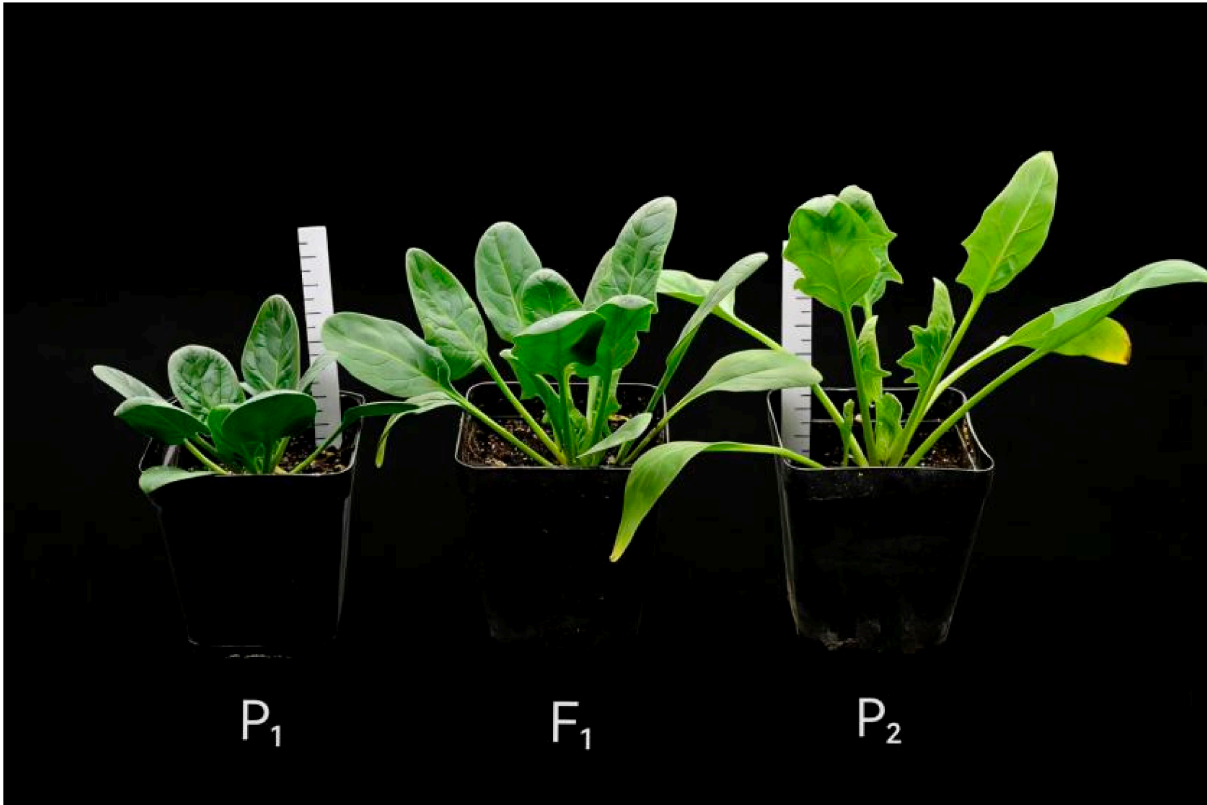


Figure 1. Blade phenotype of P₁, P₂ and F₁.

Table 2. P₁, P₂, F₁ leaf trait statistics.

Character	Greenhouse			Plastic Shed			Outdoor Nutrition Bowl		
	P ₁	P ₂	F ₁	P ₁	P ₂	F ₁	P ₁	P ₂	F ₁
Blade length (cm)	5.58	9.83	9.46	5.20	9.68	10.60	4.12	9.64	7.00
Blade width (cm)	4.78	5.33	6.04	3.56	3.98	5.43	3.16	6.28	4.80
Petiole length (cm)	2.08	7.35	4.08	2.23	6.98	7.08	0.98	4.68	2.58

Under the three cultivation modes, the average leaf length of the F₂ population ranged from 7.23 to 8.85 cm, the average leaf width ranged from 4.43 to 5.83 cm, and the average petiole length ranged from 4.27 to 5.10 cm. Among all treatments, spinach grown in outdoor nutrition bowls exhibited the minimum standard deviation of leaf width at 0.71, and the maximum coefficient of variation for petiole length across the three cultivation regimes is listed in Table 3. Leaf length, leaf width and petiole length of the F₂ population showed continuous normal or approximately normal distributions. Skewness ranged from −0.83 to 0.51, and kurtosis ranged from −0.75 to 1.31. The absolute values of all skewness were less than 3, and all kurtosis values were higher than −3, conforming to a normal distribution. Detailed frequency distribution results are presented in Figure 2.

Table 3. F₂ Population leaf trait diversity analysis.

	Greenhouse			Plastic Shed			Outdoor Nutrition Bowl		
	Blade Length	Blade Width	Petiole Length	Blade Length	Blade Width	Petiole Length	Blade Length	Blade Width	Petiole Length
Average	7.23	4.50	4.27	8.85	5.83	5.10	7.29	4.43	4.71
Maximum	12.40	8.70	10.50	13.40	10.20	11.50	11.30	7.00	9.20
Minimum	2.50	2.00	1.00	4.00	1.80	1.30	1.00	1.00	1.00
SD	1.70	1.14	1.52	1.81	1.25	1.61	1.24	0.71	1.65
CV	23.56	25.34	35.64	20.50	23.31	31.58	16.98	16.12	34.99
Skewness	0.40	0.53	0.51	−0.83	0.41	0.37	−0.21	−0.47	0.21
Kurtosis	−0.07	0.31	0.87	−0.24	−0.75	0.25	1.31	1.04	−0.48

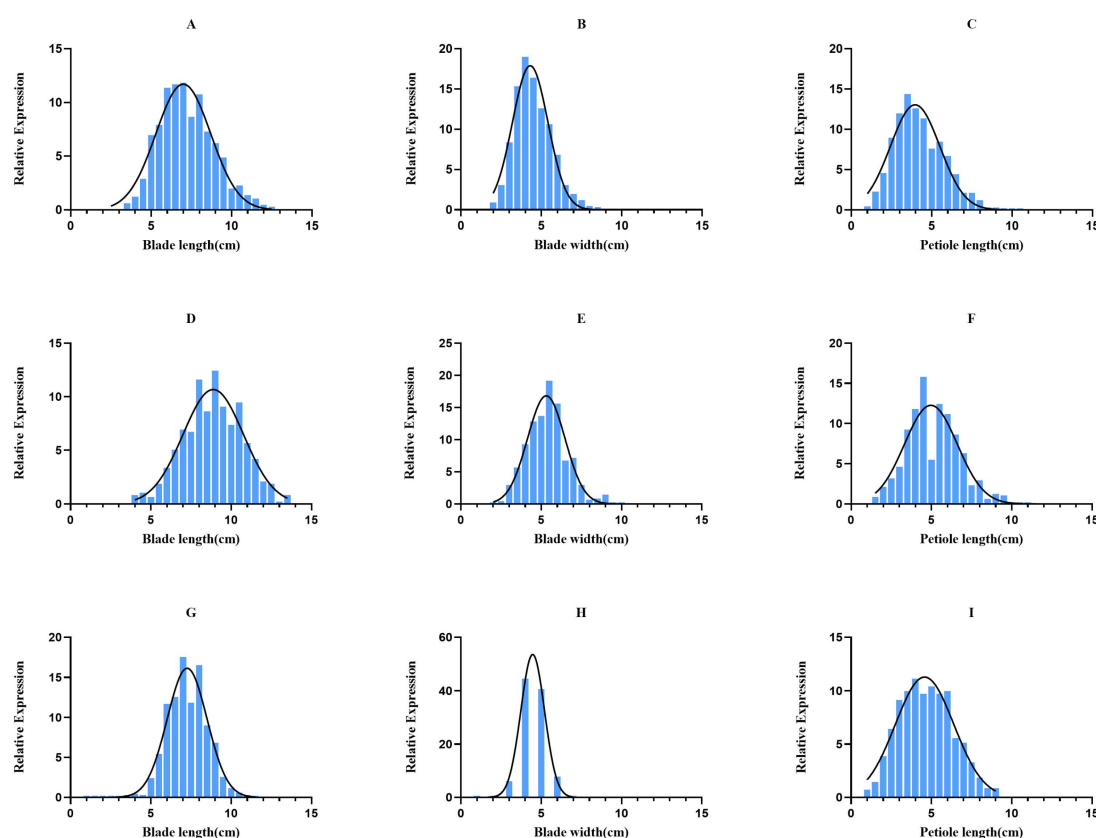


Figure 2. Frequency distributions of leaf-related traits in the F₂ population. Panels (A–C) show frequency distributions of leaf length, leaf width and petiole length for the F₂ population cultivated in the greenhouse, respectively. Panels (D–F) correspond to the three leaf traits of the F₂ population grown in the plastic shed. Panels (G–I) represent trait frequency distributions of the F₂ population cultivated in outdoor nutrition bowls.

3.2. Correlation Analysis of Leaf Type Traits

For spinach grown in the greenhouse, the correlation coefficient between leaf length and leaf width was the highest at 0.735, whereas the lowest value of 0.332 occurred between petiole length and leaf width. Under plastic shed cultivation, leaf length and leaf width had the maximum correlation coefficient of 0.759, and the minimum correlation coefficient of 0.537 was found between petiole length and leaf width. For plants cultivated in outdoor nutrition bowls, leaf length and petiole length showed the strongest correlation of 0.566, while the weakest correlation of 0.309 existed between petiole length and leaf width. Detailed results are shown in Table 4.

Table 4. Correlation analysis of leaf length, leaf width and petiole length.

	Greenhouse			Plastic Shed			Outdoor Nutrition Bowl		
	Blade Length	Blade Width	Petiole Length	Blade Length	Blade Width	Petiole Length	Blade Length	Blade Width	Petiole Length
Blade length	1.000			1.000			1.000		
Blade width	0.735	1.000		0.759	1.000		0.550	1.000	
Petiole length	0.500	0.332	1.000	0.741	0.537	1.000	0.566	0.309	1.000

3.3. Analysis of Genetic Model of Leaf Length

According to the principle of the minimum AIC (Akaike Information Criterion) value, three genetic models with low AIC values and minor differences were screened out from 24 genetic models as candidate models. The detailed results are shown in Table 5. The candidate genetic models for leaf length of spinach grown in the greenhouse were MX1-AD-ADI, MX2-ADI-ADI and MX2-ADI-AD, with corresponding AIC values of 3426.019, 3431.390 and 3435.632. The candidate genetic models for leaf length of spinach grown in the plastic shed were MX1-AD-ADI, PG-ADI and MX2-ADI-ADI, with AIC values of 2208.610, 2216.300 and 2218.521. The candidate genetic models for leaf length of spinach cultivated in outdoor nutrition bowls were MX2-A-AD, PG-ADI, and MX1-AD-ADI, and their AIC values were 2729.798, 2733.780, and 2737.780, respectively.

Table 5. Estimation of MLV and AIC values for genetic models of spinach leaf length.

	Model	MLV	AIC
Greenhouse	MX1-AD-ADI	−1701.009	3426.019
	MX2-ADI-ADI	−1697.695	3431.390
	MX2-ADI-AD	−1702.632	3435.632
Plastic shed	MX1-AD-ADI	−1092.305	2208.610
	PG-ADI	−1098.150	2216.300
	MX2-ADI-ADI	−1091.261	2218.521
Outdoor nutrition bowl	MX2-A-AD	−1355.899	2729.798
	PG-ADI	−1356.890	2733.780
	MX1-AD-ADI	−1356.890	2737.780

Note: MLV: Maximum Likelihood Value, AIC: Akaike Information Criterion.

MX1-AD-ADI was the shared genetic segregation model with the minimum AIC value among the three candidate genetic segregation models under all three cultivation modes. Two identical candidate genetic segregation models existed for leaf length between spinach grown in the greenhouse and that grown in the plastic shed: the MX1-AD-ADI model and the MX2-ADI-ADI model. Meanwhile, two shared candidate genetic segregation models for leaf length were detected between spinach cultivated in the plastic shed and those grown in outdoor nutrition bowls: the MX1-AD-ADI model and the PG-ADI model.

The candidate genetic segregation models were subjected to goodness-of-fit tests. For spinach cultivated in the greenhouse, none of the three candidate genetic segregation models for leaf length showed statistical significance; thus, MX1-AD-ADI was determined as the optimal genetic segregation model for leaf length under greenhouse cultivation. For spinach grown in the plastic shed, all three candidate genetic segregation models for leaf length failed to reach statistical significance, and MX1-AD-ADI was identified as the optimal model for leaf length in this cultivation pattern. For spinach cultivated in outdoor nutrition bowls, two statistical parameters of the MX2-A-AD model reached

significance, meaning this model was not eligible as the final optimal genetic segregation model. Neither the PG-ADI model nor the MX1-AD-ADI model exhibited statistically significant test parameters. The AIC values of the PG-ADI and MX1-AD-ADI models differed by 4.00. Considering that MX1-AD-ADI was the shared genetic segregation model across all three cultivation patterns, MX1-AD-ADI was ultimately selected as the optimal genetic segregation model for leaf length in the outdoor nutrition bowl treatment, as detailed in Table S1.

In summary, the optimal genetic segregation model controlling leaf length in spinach was MX1-AD-ADI, corresponding to a mixed inheritance model of an additive-dominant major gene plus additive-dominant-epistatic polygenes.

The first-order and second-order genetic parameters corresponding to the optimal candidate genetic segregation model under different cultivation patterns were analyzed (Table S4).

Among the first-order genetic parameters, the population mean value for leaf length of spinach cultivated in the greenhouse was 6.7699; the additive effect of the first major gene was -1.1899 , and the dominant effect of the first major gene was -1.5968 , revealing that dominance played a leading role. For spinach grown in the plastic shed, the population mean value of leaf length was 5.8166, the additive effect of the first pair of major genes was -0.6166 , and the dominant effect of the first pair of major genes was 1.7883, indicating a dominant-effect predominance. For spinach cultivated in outdoor nutrition bowls, the population mean value of leaf length was 4.2144, and the additive effect of the first pair of major genes was -0.0944 ; the dominant effect of the first major gene was 0.0083, which suggested that additive genetic effects were predominant.

Second-order genetic parameter results showed that major gene heritability for leaf length in BC_1 , BC_2 and F_2 under greenhouse cultivation was 2.96%, 43.89% and 45.17%, respectively, while polygene heritability for BC_1 , BC_2 and F_2 was 80.40%, 49.38% and 43.05%, respectively. This indicated that polygenes contributed the largest genetic effect in the BC_1 generation, and the genetic effects of major genes and polygenes were comparable in BC_2 and F_2 generations.

For spinach cultivated in the plastic shed, major gene heritability for leaf length in BC_1 , BC_2 and F_2 was 74.29%, 23.25% and 30.48%, respectively, and polygene heritability for the three generations was 15.13%, 63.79% and 63.66%, respectively. Major genes exerted the strongest genetic effect in the BC_1 generation, whereas polygenes dominated genetic variation in BC_2 and F_2 generations.

Under outdoor nutrition bowl cultivation, major gene heritability for leaf length in BC_1 , BC_2 and F_2 was 1.74%, 1.68% and 0.45%, respectively, and polygene heritability was 76.21%, 71.66% and 79.69%, respectively, demonstrating that polygenes contributed the primary genetic effect across BC_1 , BC_2 and F_2 generations.

3.4. Genetic Model Analysis of Leaf Width

According to the principle of the minimum AIC value, three genetic models with small AIC values and minor differences were screened from 24 genetic models as candidate models, as shown in Table 6. The candidate genetic segregation models for leaf width of spinach grown in the greenhouse were MX2-ADI-ADI, MX1-AD-ADI and MX2-ADI-AD, respectively, with AIC values of 2763.743, 2772.203 and 2775.383, respectively. The candidate genetic segregation models for leaf width of spinach grown in the plastic shed were 2MG-ADI, MX2-ADI-ADI, and MX1-AD-ADI, respectively, with AIC values of 1808.736, 1810.166, and 1813.526, respectively. The candidate genetic segregation models for leaf width of spinach grown in outdoor nutrition bowls were MX2-A-AD, PG-ADI, and MX1-AD-ADI, respectively, with AIC values of 1863.962, 1864.805, and 1868.806, respectively.

Table 6. Estimation of MLV and AIC values for genetic models of spinach leaf width.

	Model	MLV	AIC
Greenhouse	MX2-ADI-ADI	−1363.8720	2763.743
	MX1-AD-ADI	−1374.1010	2772.203
	MX2-ADI-AD	−1372.6920	2775.383
Plastic shed	2MG-ADI	−894.3681	1808.736
	MX2-ADI-ADI	−887.0832	1810.166
	MX1-AD-ADI	−894.7632	1813.526
Outdoor nutrition bowl	MX2-A-AD	−922.9810	1863.962
	PG-ADI	−922.4027	1864.805
	MX1-AD-ADI	−922.4029	1868.806

Note: MLV: Maximum Likelihood Value, AIC: Akaike Information Criterion.

MX1-AD-ADI was the shared genetic segregation model with the lowest AIC value among the three candidate genetic segregation models across all three cultivation modes. Two shared candidate genetic segregation models for leaf width were identified between spinach cultivated in the greenhouse and that grown in the plastic shed: MX1-AD-ADI and MX2-ADI-ADI.

Then goodness-of-fit tests were performed on the candidate models. For spinach cultivated in the greenhouse, none of the three candidate genetic segregation models for leaf width reached statistical significance. The AIC values of the MX1-AD-ADI and MX2-ADI-ADI models differed by 8.640. Since MX1-AD-ADI was the shared genetic segregation model across the three cultivation modes, MX1-AD-ADI was selected as the optimal candidate genetic segregation model for leaf width under greenhouse cultivation. For spinach grown in the plastic shed, the three candidate genetic segregation models for leaf width also failed to reach statistical significance. The AIC difference between the MX1-AD-ADI model and the 2MG-ADI model was 4.790. Given that MX1-AD-ADI was the common model for all three cultivation modes, MX1-AD-ADI was determined as the optimal candidate genetic segregation model for leaf width in the plastic shed. One out of the three candidate genetic segregation models for leaf width of spinach cultivated in outdoor nutrition bowls reached statistical significance, and the AIC values of MX1-AD-ADI and MX2-A-AD differed by 4.844. Considering MX1-AD-ADI was shared among all three cultivation patterns, MX1-AD-ADI was identified as the optimal candidate genetic segregation model for leaf width of spinach grown in outdoor nutrition bowls, as listed in Table S2.

In summary, the optimal candidate genetic segregation model governing leaf width was MX1-AD-ADI, which corresponds to a mixed inheritance model of an additive-dominant major gene plus additive-dominant-epistatic polygenes.

The first- and second-order genetic parameters of the optimal candidate genetic segregation models across various cultivation regimes were analyzed (Table S4).

Among the first-order genetic parameters, the population mean value of leaf width for spinach cultivated in the greenhouse was 5.6422. The additive effect of the first major gene was −0.8622, and the dominant effect of the first major gene was −0.9179, revealing that genetic variation was mainly controlled by dominant effects. The population mean value of leaf width for spinach grown in the plastic shed was 4.2775. The additive effect of the first pair of major genes was −0.7175, and the dominant effect of the first major gene was −0.7523, indicating a predominance of dominant genetic effects. The population mean value of leaf width for spinach grown in outdoor nutrition bowls was 3.1972. The additive

effect of the first major gene was -0.0372 , and the dominant effect of the first major gene was 0.0017 , demonstrating that additive effects dominated genetic variation.

Second-order genetic parameter results showed that major gene heritability for leaf width in BC_1 , BC_2 and F_2 of spinach under greenhouse cultivation was 1.01% , 29.44% and 42.87% , respectively, while polygene heritability for BC_1 , BC_2 and F_2 was 86.06% , 64.95% and 43.15% , respectively. This suggested that polygenes contributed the largest genetic effect across BC_1 , BC_2 and F_2 generations.

For spinach cultivated in the plastic shed, major gene heritability for leaf width in BC_1 , BC_2 and F_2 was 2.62% , 40.36% and 24.68% , respectively, and polygene heritability for the three generations was 74.75% , 51.48% and 67.35% , respectively, indicating that polygenes exerted the primary genetic effect in BC_1 , BC_2 and F_2 generations.

Under outdoor nutrition bowl cultivation, major gene heritability for leaf width in BC_1 , BC_2 and F_2 was 1.65% , 1.55% and 0.31% , respectively, and polygene heritability was 66.86% , 81.29% and 70.57% , respectively, which illustrated that polygenes contributed the predominant genetic effect in BC_1 , BC_2 and F_2 generations.

3.5. Genetic Model Analysis of Petiole Length

According to the principle of the minimum AIC value, three genetic models with small AIC values and minor differences were screened from 24 genetic models as candidate models, as shown in Table 7. The candidate genetic segregation models for petiole length of spinach grown in the greenhouse were MX1-AD-ADI, MX2-ADI-AD and 2MG-AD, respectively, with AIC values of 3173.305, 3173.460 and 3173.480, respectively. The candidate genetic segregation models for petiole length of spinach grown in the plastic shed were MX2-A-AD, MX2-ADI-AD, and PG-ADI, respectively, with AIC values of 2082.589, 2087.501, and 2097.831, respectively. The candidate genetic segregation models for petiole length of spinach grown in outdoor nutrition bowls were MX2-ADI-ADI, MX2-ADI-AD and 2MG-ADI, respectively, with AIC values of 3105.550, 3107.860 and 3116.587, respectively.

Table 7. Estimation of MLV and AIC values for genetic models of spinach petiole length.

	Model	MLV	AIC
Greenhouse	MX1-AD-ADI	-1574.653	3173.305
	MX2-ADI-AD	-1571.730	3173.460
	2MG-AD	-1576.74	3173.480
Plastic shed	MX2-A-AD	-1032.295	2082.589
	MX2-ADI-AD	-1028.751	2087.501
	PG-ADI	-1038.915	2097.831
Outdoor nutrition bowl	MX2-ADI-ADI	-1534.775	3105.550
	MX2-ADI-AD	-1538.930	3107.860
	2MG-ADI	-1548.294	3116.587

Note: MLV: Maximum Likelihood Value, AIC: Akaike Information Criterion.

MX1-AD-ADI was the shared genetic segregation model with the lowest AIC value among the three candidate genetic segregation models across all three cultivation modes.

Then goodness-of-fit tests were performed on the candidate models. Under greenhouse cultivation, none of the three candidate genetic segregation models for spinach petiole length reached statistical significance, and the AIC values of the MX2-ADI-AD and MX1-AD-ADI models differed by 0.155 . Since MX2-ADI-AD was the shared genetic segregation model across the three cultivation modes, MX2-ADI-AD was iden-

tified as the optimal candidate genetic segregation model for petiole length under greenhouse cultivation.

For plastic shed cultivation, one statistical parameter of both the MX2-A-AD model and the PG-ADI model reached significance, so neither could be selected as the final candidate genetic segregation model; meanwhile, the MX2-ADI-AD model failed to reach statistical significance. Consequently, MX2-ADI-AD was determined as the optimal candidate genetic segregation model for petiole length in plastic shed cultivation.

For spinach cultivated in outdoor nutrition bowls, neither the MX2-ADI-AD model nor the MX2-ADI-ADI model for petiole length reached statistical significance. Four statistical parameters of the 2MG-ADI model reached significance, meaning this model was ineligible to serve as the final candidate genetic segregation model, and the AIC values between the MX2-ADI-AD and MX2-ADI-ADI models differed by 2.310. Given that MX2-ADI-AD was the common genetic segregation model for all three cultivation modes, MX2-ADI-AD was selected as the optimal candidate genetic segregation model for petiole length under outdoor nutrition bowl cultivation, as detailed in Table S3.

In summary, the optimal candidate genetic segregation model controlling petiole length was MX2-ADI-AD, corresponding to a mixed inheritance model containing two pairs of additive-dominant-epistatic major genes plus additive-dominant polygenes.

The first-order and second-order genetic parameters corresponding to the optimal candidate genetic segregation model under different cultivation modes were analyzed (Table S4).

Among the first-order genetic parameters, the population mean value of petiole length for spinach cultivated in the greenhouse was 3.4768. The additive effect values of the first and second major genes were both -0.9652 ; the dominant effect values of the first and second major genes were 0.9270 and 0.4664 , respectively. The polygenic additive effect was -0.6904 , and the polygenic dominant effect was 1.3475 , revealing that genetic variation was predominantly controlled by dominant effects. The population mean value of petiole length for spinach cultivated in the plastic shed was 4.2743. The additive effect values of the first pair and second pair of major genes were both -1.1664 ; the dominant effect values of the first pair and second pair of major genes were -1.3557 and -0.4315 , respectively. The polygenic additive effect was -0.0394 , and the polygenic dominant effect was 2.1379 , indicating a predominance of dominant genetic effects. The population mean value of petiole length for spinach grown in outdoor nutrition bowls was 5.3949, and the additive effect values of the first pair and the second pair of major genes were both 0.1179 . The dominant effect values of the first pair and the second pair of major genes were -0.0539 and 0.3082 , respectively; the polygenic additive effect was -2.3883 , and the polygenic dominant effect was -0.9610 , demonstrating that dominant effects dominated genetic variation.

Second-order genetic parameter results showed that major gene heritability for petiole length in BC_1 , BC_2 and F_2 of spinach under greenhouse cultivation was 37.35%, 61.45% and 66.30%, respectively, while polygene heritability for BC_1 , BC_2 and F_2 was 36.63%, 24.65% and 16.80%, respectively. This indicated that major genes contributed the largest genetic effect across BC_1 , BC_2 and F_2 generations.

For spinach cultivated in the plastic shed, major gene heritability for petiole length in BC_1 , BC_2 and F_2 was 70.12%, 74.71% and 61.81%, respectively, and polygene heritability for the three generations was 18.75%, 13.38% and 31.37%, respectively, showing that major genes exerted the predominant genetic effect in BC_1 , BC_2 and F_2 generations.

Under outdoor nutrition bowl cultivation, major gene heritability for petiole length in BC_1 , BC_2 and F_2 was 74.57%, 61.86% and 76.47%, respectively, and polygene heritability was 6.46%, 0.00% and 11.36%, respectively, which illustrated that major genes had the greatest genetic effect in BC_1 , BC_2 and F_2 generations.

3.6. Construction of High-Density Genetic Map of Spinach

The two parents and 152 F₂ individuals were subjected to whole-genome resequencing. The P₁ and P₂ samples generated 18.6 million and 22.72 million clean reads (counted separately for paired-end Read 1 and Read 2), corresponding to 27.19 Gb and 33.99 Gb of sequencing data, respectively. A total of 468.99 Gb of sequencing data was obtained from the 152 F₂ individuals, with an average Q30 value of 93.34% (Table 8). The average mapping rate of clean reads for each F₂ individual reached 98.06% (Table 9). The average sequencing depth for both parental lines exceeded 20×, with a genome coverage ratio (regions covered by at least 1× reads) higher than 90%. The average sequencing depth of F₂ samples was 3.45×, and their genome coverage ratio (≥1×) was greater than 78.12% (Table 10).

Table 8. Statistics of sequencing data of samples.

Sample ID	Total Clean Reads	Total Clean Bases	Q30 Percentage (%)	GC Percentage (%)
P ₁	181,697,782	27,190,395,154	95.84	95.84
P ₂	227,264,922	33,991,179,578	96.85	96.85
F ₂	3,138,450,390	468,989,454,122	93.34	93.34
Total	3,547,413,094	530,171,028,854	93.38	93.38

Note: Sample ID: P1 represents the female parent, and P2 represents the male parent. Total clean reads: statistics of clean reads for each end of paired-end sequencing. Total clean bases: total amount of filtered bases for each sample. Q30 percentage: proportion of bases with sequencing quality value ≥ 30. GC percentage: proportion of G and C bases among all sequenced bases. F₂: total or average value of sequencing data for F₂ progeny. Total: overall summary of all sequencing data.

Table 9. Statistical table of comparison results of each sample.

Sample ID	Clean Reads	Mapped (%)	Properly_Mapped (%)
P ₁	181,697,782	99.33	95.29
P ₂	227,264,922	99.52	95.52
F ₂ (average)	20,512,700	98.06	91.75

Note: Sample ID: P1 represents the female parent, and P2 represents the male parent. Clean reads: total number of clean reads, counted separately for paired-end Read 1 and Read 2 (Read 1 and Read 2 are each counted as one read). Mapped (%): proportion of clean reads aligned to the reference genome relative to all clean reads. Properly_mapped (%): proportion of paired-end reads with both ends aligned to the reference genome and falling within the expected insert size range. F₂ (average): average value of corresponding indicators across all F₂ progeny samples.

Table 10. Sample coverage depth and coverage percentage statistics.

Sample ID	Ave-Depth	Cov_Ratio_1× (%)	Cov_Ratio_5× (%)	Cov_Ratio_10× (%)
P ₁	30.00	98.27	96.26	93.24
P ₂	37.00	98.56	96.75	94.33
F ₂ (average)	3.45	78.12	23.40	3.30

Note: Sample ID: P1 represents the female parent, and P2 represents the male parent. Ave-depth: average sequencing depth per sample. Cov_ratio: the proportion of genomic bases covered at or above a specified depth relative to the total bases of the reference genome.

SNP markers were developed, yielding a total of 1,382,243 SNPs that were assigned to six linkage groups. These SNPs were used to generate bin markers, and bin markers shorter than 5 kb were retained. Segregation distortion filtering was conducted to remove polymorphic markers with severe segregation distortion (Chi-square test $p < 0.0001$). After screening, 13,545 filtered bin markers were retained.

Using HighMap software (Beijing Biomarker Technology Co., Ltd. Beijing, China) [43], we repeatedly eliminated redundant markers and sorted the remaining 13,545 bin markers. The software calculated optimal recombination frequencies between markers to optimize

marker ordering, and 5900 valid bin markers were finally obtained for high-density genetic map construction.

These bin markers were classified into six linkage groups. HighMap analysis determined the linear order of markers within each linkage group and estimated genetic distances between adjacent markers; a genetic map with a total genetic length of 843.48 cM was ultimately constructed. The lengths of the six linkage groups ranged from 103.76 to 179.88 cM. The fourth linkage group contained the largest number of total bin markers at 1188, while the third linkage group had the fewest bin markers with a count of 544. The fourth linkage group had the longest total genetic distance of 179.88 cM, and the fifth linkage group had the shortest total genetic distance at 103.76 cM. The third linkage group exhibited the largest average marker interval of 0.25 cM, and the fifth linkage group showed the smallest average interval at 0.10 cM. The maximum gap on the first linkage group reached 7.09 cM, whereas the sixth linkage group had the smallest maximum gap, which was 1.64 cM. The proportion of intervals smaller than 5 cM across the six linkage groups ranged from 99.82% to 100.00%, as shown in Table 11.

Table 11. Basic information on genetic mapping.

LG	Total Bin Marker	Total Distance (cM)	Average Distance (cM)	Max Gap (cM)	Gaps < 5 cM (%)
LG01	1187	157.54	0.13	7.09	99.83%
LG02	906	114.71	0.13	5.62	99.89%
LG03	544	135.67	0.25	5.16	99.82%
LG04	1188	179.88	0.15	4.88	100.00%
LG05	1043	103.76	0.10	1.65	100.00%
LG06	1032	151.93	0.15	1.64	100.00%
Total	5900	843.48	0.14	7.09	99.93%

Note: LG: linkage group. Total bin markers: total number of bin markers on each linkage group, and the Total row indicates the cumulative number of markers across all linkage groups. Total distance (cM): total genetic length of each linkage group, and the Total row indicates the sum of genetic lengths of all linkage groups. Average distance (cM): average marker interval of each linkage group, and the Total row represents the average interval value calculated across all linkage groups. Max gap (cM): the largest marker interval within each linkage group, and the Total row indicates the maximum gap value among all linkage groups. Gap < 5 cM (%): percentage of marker intervals shorter than 5 cM, and the Total row presents the aggregated value for all linkage groups.

3.7. QTL Analysis of Leaf Type

QTL mapping for leaf-related traits in the F₂ population was performed using MapChart 2.32 software. One QTL controlling leaf length, designated qLL2-1, was mapped onto chromosome 2. Two QTLs associated with leaf width, namely qLW1-1 and qLW2-1, were located on chromosome 1 and chromosome 2, respectively. One QTL for petiole length was detected on chromosome 2 and named qPL2-1, as illustrated in Figure 3.

The candidate interval for the spinach leaf-length QTL qLL2-1 spanned between bin markers Block6089 and Block6094, with an LOD score of 6.767, an additive effect of −0.792, and a phenotypic variance explained (PVE) of 17.48%. The candidate intervals for leaf-width QTLs qLW1-1 and qLW2-1 were located between bin markers Block2973 and Block2976, and between bin markers Block12977 and Block12984, respectively. Their LOD values were both 3.000, additive effects were −0.229 and −0.257, and phenotypic variance-explained (PVE) values were 4.44% and 6.99%, respectively. The candidate interval for the spinach petiole-length QTL qPL2-1 was positioned between bin markers Block5449 and Block5457, with an LOD value of 6.588, an additive effect of −0.842, and a phenotypic variance-explained value of 17.26%, as shown in Table 12.

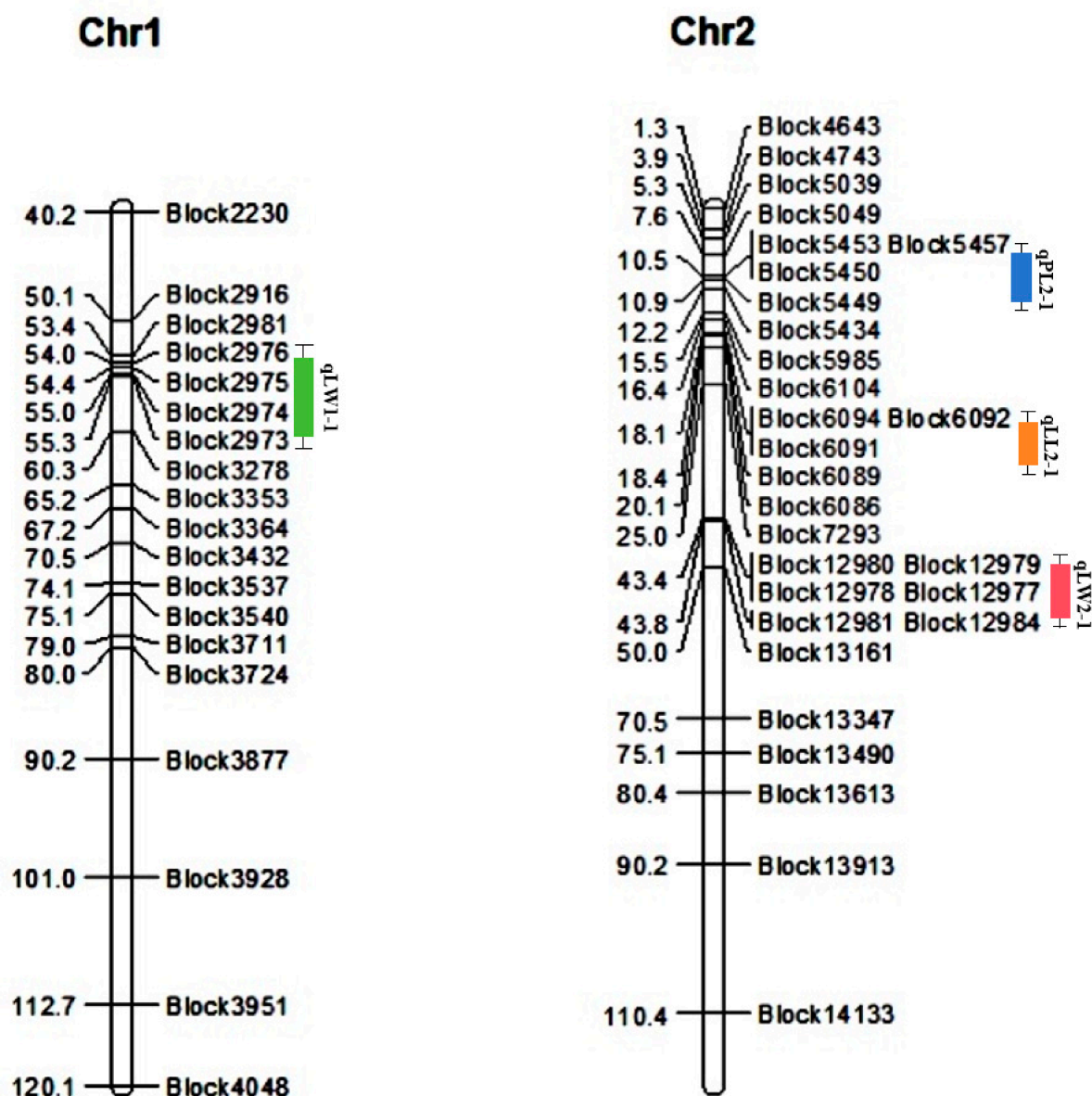


Figure 3. Distribution of QTLs controlling leaf-type traits on chromosomes in the F₂ population. Chr1: chromosome 1; Chr2: chromosome 2; green: QTL for leaf width located on chromosome 1; blue: QTL for petiole length located on chromosome 2; orange: QTL for leaf length located on chromosome 2; red: QTL for leaf width located on chromosome 2.

Table 12. Spinach leaf trait QTLs.

Trait	QTL Name	Chr	Flanking Markers	LOD	ADD	DOM	PVE
Blade length	qLL2-1	2	Block6089-Block6094	6.767	−0.792	0.451	17.48
Blade width	qLW1-1	1	Block2973-Block2976	3.000	−0.229	−0.013	4.44
Blade width	qLW2-1	2	Block12977-Block12984	3.000	−0.257	−0.179	6.99
Petiole length	qPL2-1	2	Block5449-Block5457	6.588	−0.842	0.768	17.26

Note: Trait: name of the measured trait. QTL name: designated name of each QTL. Chr: corresponding linkage group number. Flanking markers: positions of flanking tag markers. LOD: logarithm of odds; ADD: additive effect; DOM: dominant effect; PVE: phenotypic variance explained.

3.8. Determination of Candidate Genes

3.8.1. Candidate Genes for Leaf Length and Leaf Width

No candidate genes were identified within the candidate interval for the leaf-length QTL qLL2-1 in spinach. The two leaf-width QTLs qLW1-1 and qLW2-1 contained two and 13 genes in their respective intervals, as summarized in Table 13. These 15 genes were annotated against the spinach reference gene database. According to functional annotation data from the SpinachBase database, *SOV1g017270* primarily functioned in cell cycle regulation, chromosome stability and polyamine metabolism to maintain genomic stability. *SOV2g023070* functioned in single-stranded DNA binding, participating in DNA replication, repair and recombination to preserve genome stability. *SOV2g023120* catalyzed phenylalanine biosynthesis and was involved in the production of aromatic amino acids and secondary metabolites. *SOV2g023110* participated in abiotic stress responses and improved plant stress tolerance. *SOV2g023150* eliminated reactive oxygen species such as hydrogen peroxide, alleviated oxidative stress, and protected chloroplasts and cellular structures. *SOV2g023160* bound RNA and took part in RNA processing, stabilization and translational regulation. *SOV2g023060* encoded a protein with unknown function. *SOV2g023170* interacted with the small GTPase ROP to regulate cytoskeleton dynamics and signal transduction. *SOV2g023100* mediated retrotransposon transposition via reverse transcriptase and integrase, thereby affecting genomic structure and evolution. Based on their annotated functions, we speculated that these nine genes were unlikely to participate in spinach leaf development.

Table 13. Spinach leaf width and petiole length candidate interval function notes.

	Gene ID	Chr	nr_Annotation
Blade width	<i>SOV1g017270</i>	<i>SOVchr1</i>	Polyamine-modulated factor 1-binding protein [Spinacia oleracea]
	<i>SOV1g017280</i>	<i>SOVchr1</i>	Zinc finger FYVE domain protein [Spinacia oleracea]
	<i>SOV2g023070</i>	<i>SOVchr2</i>	Replication protein A subunit [Spinacia oleracea]
	<i>SOV2g023120</i>	<i>SOVchr2</i>	Arogenate dehydratase [Spinacia oleracea]
	<i>SOV2g023110</i>	<i>SOVchr2</i>	Stress response protein NST1 [Spinacia oleracea]
	<i>SOV2g023090</i>	<i>SOVchr2</i>	Serine/threonine-protein phosphatase7 long form-like [Spinacia oleracea]
	<i>SOV2g023150</i>	<i>SOVchr2</i>	Ascorbate peroxidase [Spinacia oleracea]
	<i>SOV2g023160</i>	<i>SOVchr2</i>	S1 domain-containing protein [Spinacia oleracea]
	<i>SOV2g023140</i>	<i>SOVchr2</i>	Protein PIN-LIKES 2 [Spinacia oleracea]
	<i>SOV2g023050</i>	<i>SOVchr2</i>	E3 ubiquitin-protein ligase [Spinacia oleracea]
	<i>SOV2g023060</i>	<i>SOVchr2</i>	Unknown protein [Spinacia oleracea]
	<i>SOV2g023170</i>	<i>SOVchr2</i>	Interactor of constitutive active ROPs 3-like protein [Spinacia oleracea]
	<i>SOV2g023130</i>	<i>SOVchr2</i>	Mitogen-activated protein kinase [Spinacia oleracea]
	<i>SOV2g023080</i>	<i>SOVchr2</i>	Putative DDB1-and CUL4-associated factor 8 [Spinacia oleracea]
	<i>SOV2g023100</i>	<i>SOVchr2</i>	Retrovirus-related Pol polyprotein from transposon TNT 1-94 [Spinacia oleracea]
Petiole Length	<i>SOV2g003700</i>	<i>SOVchr2</i>	Unknown protein [Spinacia oleracea]
	<i>SOV2g003710</i>	<i>SOVchr2</i>	FAR1 domain-containing protein [Spinacia oleracea]
	<i>SOV2g003720</i>	<i>SOVchr2</i>	ULP_PROTEASE domain-containing protein [Spinacia oleracea]
	<i>SOV2g003690</i>	<i>SOVchr2</i>	zf-GRF domain-containing protein [Spinacia oleracea]

Gene ID represents the gene identifier, Chr denotes the chromosome where the gene is located, nr_annotation refers to the functional annotation of genes.

Combined with gene functional annotation, the remaining six genes potentially associated with leaf development, namely *SOV1g017280*, *SOV2g023090*, *SOV2g023140*, *SOV2g023050*, *SOV2g023130*, and *SOV2g023080*, were regarded as candidate genes regulating spinach leaf width. The expression patterns of these six genes were quantified in the two parental lines P_1 and P_2 . The results showed that the transcript abundance of *SOV1g017280* and *SOV2g023050* exhibited no significant difference between the two parents. The expression levels of *SOV2g023090*, *SOV2g023130* and *SOV2g023080* were significantly higher in P_1 than those in P_2 . The transcript level of *SOV2g023140* was also significantly higher in P_1 relative to P_2 , as presented in Figure 4. *SOV2g023090* modulated protein activity through dephosphorylation and participated in signal transduction as well as growth and developmental regulation. *SOV2g023130* encoded a core signaling kinase that transmitted signals related to stress, phytohormones, growth and development to modulate downstream gene expression. *SOV2g023080* functioned in the ubiquitin–proteasome pathway, governing protein degradation, DNA repair and developmental processes. *SOV2g023140* encodes a putative *PIN* auxin transporter that participates in auxin transport and distribution, further regulating plant organ formation and polar growth. Taken together, *SOV2g023140* was proposed as the key candidate gene responsible for leaf width regulation in spinach, and was designated *SpPINL2*.

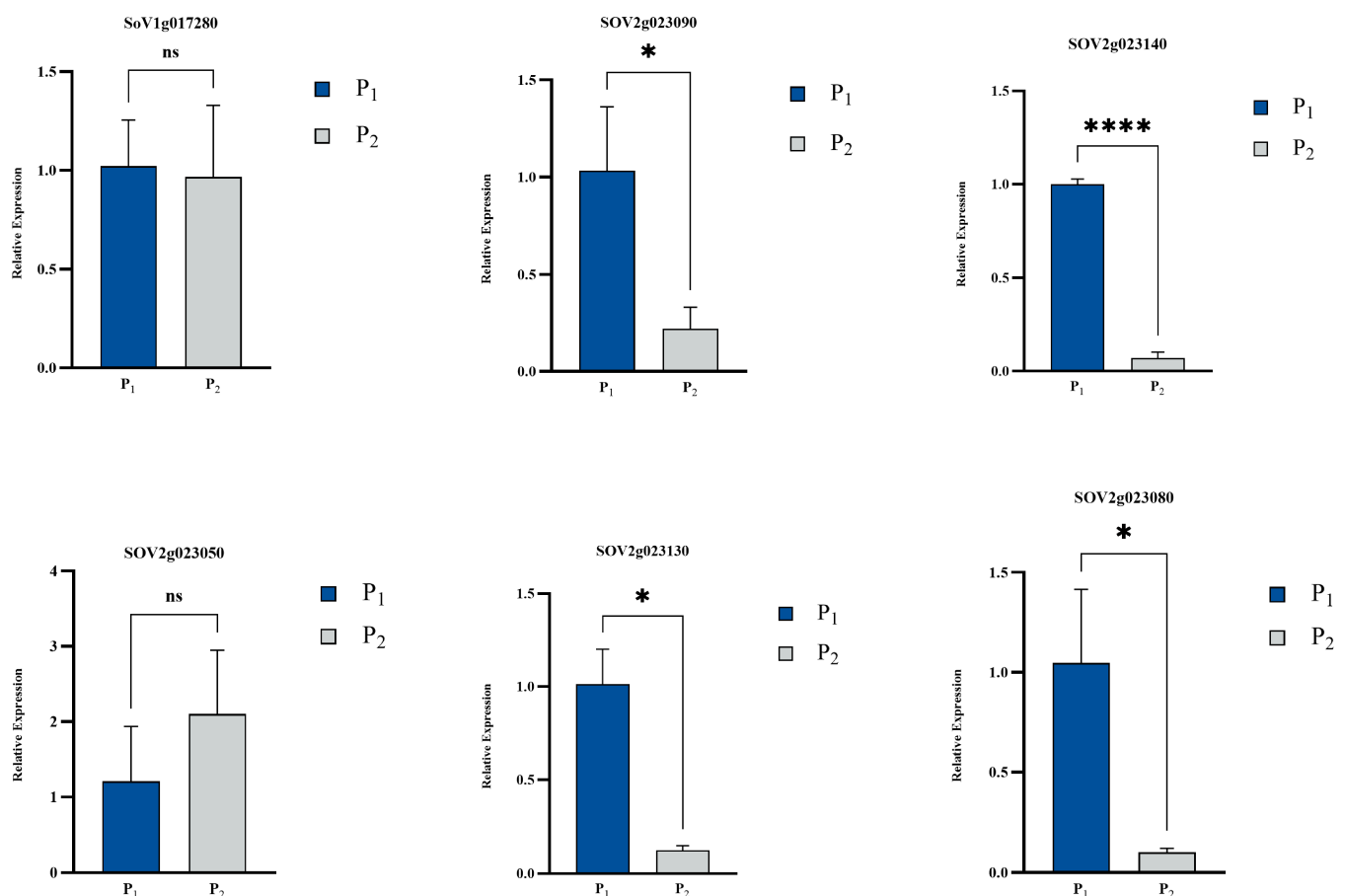


Figure 4. Expression levels of leaf width candidate genes in P_1 and P_2 . Blue: expression level of female parent P_1 ; gray: expression level of male parent P_2 ; ns: non-significant difference; *: $p < 0.05$, significant difference; ****: $p < 0.0001$, extremely significant difference.

3.8.2. Candidate Genes for Petiole Length

Four genes were located within the candidate interval for the spinach petiole-length QTL (qPL2-1). According to gene annotation results, *SOV2g003700* encoded a protein

with unknown function. The main function of *SOV2g003710* was to participate in the regulation of light signaling, flowering time and plant growth and development. The main function of *SOV2g003720* was to mediate the removal of *SUMO* modifications and regulate protein localization, protein stability and signaling pathways. *SOV2g003690* was a zinc finger nucleic acid-binding protein that participated in transcriptional regulation, RNA binding, and the regulation of growth and development. Among these genes, *SOV2g003710*, *SOV2g003720* and *SOV2g003690* formed a “*FAR1-ULP-zf-GRF*” gene cluster. Expression levels of the four genes were quantified in the two parental lines. The results showed that *SOV2g003690* was not expressed in either parent; *SOV2g003700* showed no significant difference in expression between the two parents. The expression level of *SOV2g003720* in P1 was significantly higher than that in P2, while the expression level of *SOV2g003710* in P1 was extremely significantly higher than that in P2, as shown in Figure 5. Therefore, this gene cluster was selected as a candidate gene cluster regulating petiole length in spinach and named *SpFAR1-ULP-zf-GRF*.

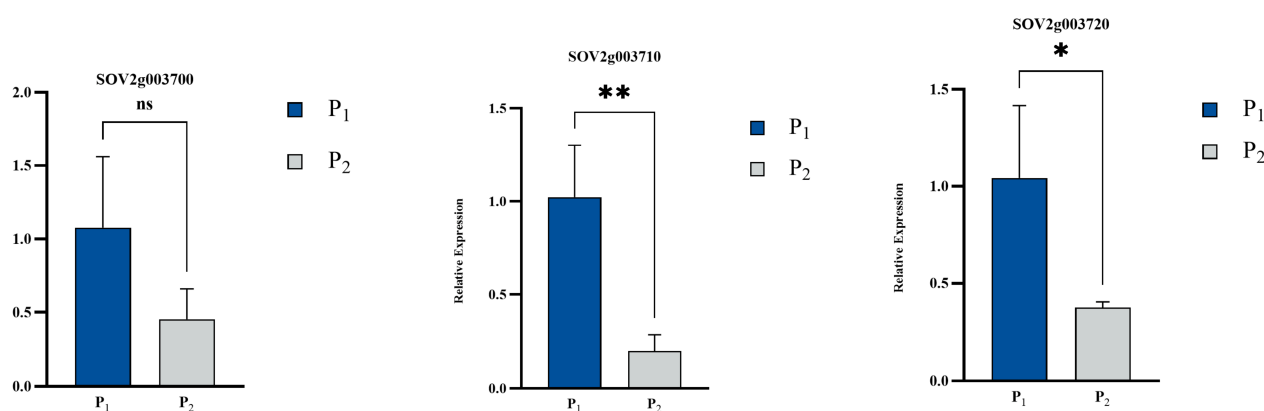


Figure 5. Expression levels of petiole length candidate genes in P1 and P2. Blue: expression level of female parent P1; gray: expression level of male parent P2; ns: non-significant difference; *: $p < 0.05$, significant difference; **: $p < 0.01$, highly significant difference.

4. Discussion

Different cultivation patterns exert substantial effects on the phenotypes of arugula and Chinese cabbage [44,45]. In the present study, leaf length and petiole length of spinach grown in outdoor nutrition bowls were longer than those of spinach cultivated in the greenhouse; meanwhile, leaf width of spinach grown in outdoor nutrition bowls was greater than that of greenhouse-grown spinach. We innovated and revised the criteria for selecting the optimal genetic segregation model: instead of merely adopting the rule of the smallest AIC value paired with goodness-of-fit test deviation, we applied a comprehensive criterion that integrates AIC values and minor goodness-of-fit deviations. Using this strategy, the shared optimal genetic segregation models governing spinach leaf traits were identified across the three cultivation modes. The optimal model for leaf length was MX1-AD-ADI, for leaf width was MX1-AD-ADI, and for petiole length was MX2-ADI-AD. The identification of consistent genetic models across divergent cultivation environments minimized the interfering effects caused by different cultivation conditions. Such shared genetic models are therefore more reliable than models obtained from only a single cultivation pattern. The above genetic model also provides a theoretical basis for germplasm innovation in spinach.

During spinach breeding, petiole length receives greater attention than other leaf-related traits, and it serves as one of the key indicators for breeders to assess the DUS characteristics of spinach varieties. Previous studies also verified a significant positive correlation between leaf length and leaf width, while no significant correlation was detected

between leaf length and petiole length in spinach [46,47]. Our results indicate that petiole length had the largest coefficient of variation among the measured spinach traits; leaf length and leaf width were strongly correlated, whereas the correlation between leaf length and petiole length was weak. These findings provide a theoretical basis highlighting the importance of petiole length selection in spinach breeding programs.

Genotype–environment interaction is ubiquitous in plants, describing the differential phenotypic responses of distinct genotypes when exposed to varied environmental conditions [48]. Such interactions constitute a major source of phenotypic variation, adaptive differentiation, and trait instability across heterogeneous environments. With advances in quantitative genetics and genomics, accumulating studies have demonstrated that plant growth, development and agronomic traits are generally modulated by genetic loci together with external environments. Gene–environment interactions broadly regulate plant responses to stress, photoperiod and climatic cues, and substantially shape phenotypic plasticity [49,50]. Meanwhile, numerous QTL mapping and multi-environment trials in crop genetics and breeding have validated gene–environment interactions, laying a theoretical foundation for dissecting complex genetic traits and enhancing cultivar adaptability [51,52]. Environmental conditions can alter gene heritability, and divergent environments exert distinct effects on genetic variances and phenotypic variances [53–56]. In the present study, identical optimal genetic models were detected across different cultivation patterns; nevertheless, estimation of first- and second-order genetic parameters within these shared models revealed significant differences in major gene and polygene genetic parameters under alternative cultivation regimes. These discrepancies may stem from environmental regulation on the function and expression of relevant genes.

To clarify the novelty of leaf-shape QTLs identified in the present study, we compared the physical intervals of leaf-morphology-associated QTLs mapped on spinach chromosomes 1 and 2 in our research with the leaf-shape QTL mapping results published in 2021. Using a BC₁ population, four QTLs for leaf length distributed on linkage groups 3 and 5, three QTLs for leaf width all located on linkage group 3, and two QTLs for petiole length mapped on linkage group 5 were reported. For the QTLs controlling leaf length and petiole length in that experiment, no data are available to map their linkage groups to corresponding chromosomal positions. The QTLs for leaf width identified in that study were also located on chromosome 1, yet within distinct intervals compared with those detected in the present experiment.

The candidate interval of qLL2-1 controlling leaf length in this study resides on chromosome 2 with a relatively high phenotypic contribution rate of 17.48%. Notably, no annotated genes were found within this interval, implying that this region may be tightly linked to the causal candidate genes regulating spinach leaf length. Subsequent screening of candidate genes for spinach leaf length should focus on genes upstream and downstream of this genomic region. The candidate intervals of leaf-width QTLs qLW1-1 and qLW2-1 are located on chromosomes 1 and 2, respectively, while the petiole-length QTL qPL2-1 was anchored to chromosome 2.

PIN proteins mediate polar auxin asymmetric distribution via their polar subcellular localization, thereby participating in numerous biological processes that modulate plant growth and development. The *PIN* gene family is highly conserved and exhibits species-specific characteristics across plants. Eight *PIN* members have been identified in *Arabidopsis thaliana*, with obvious divergence in subcellular localization and biological function. In addition, variable numbers of *PIN* homologous genes have been successively characterized in crops including rice, maize and sorghum [57,58]. In the present study, a candidate gene *SpPINL2* (SOV2g023140) related to the regulation of spinach leaf width was

screened. The expression level of this gene was extremely significantly higher in P_1 than that in P_2 , implying its potential involvement in spinach leaf width development.

The plant-specific synergistic functional gene cluster *FAR1-ULP-zf-GRF* widely exists in *Arabidopsis thaliana*, rice, maize and other plant species. Within this cluster, *FAR1*, *ULP* and *zf-GRF* individually participate in light signaling pathways, *SUMO* modification of proteins, and regulation of organ growth and cell proliferation, respectively. Multiple genes synergistically regulate diverse plant growth and developmental processes [32,39,59,60]. In this study, a three-gene *FAR1-ULP-zf-GRF* cluster was mapped to chromosome 2 in spinach. The *zf-GRF* gene (SOV2g003690) showed no detectable expression in either parent, while the expression levels of *FAR1* (SOV2g003710) and *ULP* (SOV2g003720) were significantly higher in P_1 than those in P_2 . We speculated that *FAR1* (SOV2g003710), *ULP* (SOV2g003720) and *zf-GRF* (SOV2g003690) jointly constitute the *FAR1-ULP-zf-GRF* gene cluster in spinach to regulate petiole elongation. Nevertheless, the exact biological function of this cluster requires further experimental validation in follow-up research.

5. Conclusions

The optimal genetic model for leaf length and leaf width was MX1-AD-ADI, and for petiole length was MX2-ADI-AD. A high-density genetic map containing 5900 bin markers across six linkage groups with a total genetic length of 843.48 cM was constructed using an F2 segregating population. One leaf-length QTL qLL-1 was mapped to chromosome 2 with no candidate genes annotated in its interval; two leaf-width QTLs qLW1-1 and qLW2-1 harbored 15 total candidate genes, among which SOV2g023140 was identified as the key regulatory gene and designated SpPINL2. The petiole-length QTL qPL2-1 contained four genes forming a *FAR1-ULP-zf-GRF* cluster named Sp*FAR1-ULP-zf-GRF* after combined genome annotation and expression analysis.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12070875/s1>. Table S1: MX1-AD-ADI Model Goodness-of-Fit Test for Spinach Leaf Length. Table S2: MX1-AD-ADI Model Goodness-of-Fit Test for Spinach Leaf Width. Table S3: MX2-ADI-AD Model Goodness-of-Fit Test for Spinach Petiole Length. Table S4: Estimates of Genetic Parameters for Leaf Length, Leaf Width and Petiole Length in Spinach.

Author Contributions: Conceptualization, L.H. and Z.Q.; methodology, Z.Q.; software, L.H.; validation, L.H., Y.J. and S.Y.; formal analysis, L.H.; investigation, L.H.; resources, Z.Q.; data curation, L.H.; writing—original draft preparation, L.H.; writing—review and editing, S.Y. and Z.Q.; visualization, Z.Q.; supervision, Z.Q.; project administration, Z.Q.; funding acquisition, Y.J. and Z. Q. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Development Project of Vegetable Research Institute, BAAFS, grant number KYCX202603; Innovation and Capacity-Building Project of BAAFS, grant number KJCX20261416; China Agriculture Research System of MOF and MARA, CARS—Major Vegetables, grant number CARS-21.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Acknowledgments: We sincerely appreciate the help provided by all members of the Vegetable Research Center, Beijing Academy of Agriculture and Forestry Sciences in this work.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

MLV	Maximum Likelihood Value
AIC	Akaike Information Criterion
LOD	Logarithm of Odds
ADD	Additive Effect
DOM	Dominant Effect
PVE	Phenotypic Variance Explained
MX1-AD-ADI	A mixed genetic model of additive-dominant major gene + additive-dominant-epistatic polygenes
MX2-ADI-ADI	Two pairs of additive-dominant-epistatic major gene + additive-dominant-epistatic polygene mixed genetic model
MX2-ADI-AD	Two pairs of additive-dominant-epistatic major gene + additive-dominant polygene mixed genetic model
MX2-A-AD	Two pairs of additive-additive epistatic major gene + additive-dominant polygene mixed genetic model
PG-ADI	Additive-dominance-epistatic pure polygene genetic model
2MG-ADI	Two pairs of additive-dominant-epistatic major gene models
2MG-AD	Two pairs of additive-dominant major gene models

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