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ASSESSING ORIGIN AND GENETIC DIVERSITY OF CHONGMING SPINACH BASED ON MORPHOLOGICAL AND SSR MARKERS

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ABSTRACT

Spinach (Spinacia oleracea) is an important leafy vegetable that is rich in essential nutrients. In this study, 42 spinach accessions from various countries and regions were analyzed to assess their genetic diversity and phylogenetic relationships using morphological traits and SSR markers. A total of 198 alleles were amplified with 57 pairs of SSR primers, demonstrating a high level of polymorphism. Based on SSR data, the accessions were clustered into two major groups: Clade I consisted mainly of accessions from northern China, Russia, Europe, and America, while Clade II included accessions from Shanghai, southern China, and East Asia. Notably, accessions accessions from northern and southern China were clearly separated, suggesting distinct genetic origins. Furthermore, a fingerprinting database was established using nine core SSR markers. Among these, three primers (primer 9, 40, and 46) successfully distinguished three Chongming local accessions (B26, B35, and B40). This study provides insights into the genetic diversity and geographical differentiation of spinach germplasm, thereby supporting conservation efforts for local varieties and facilitating future spinach breeding programs.

KEYWORDS: Spinacia oleracea; morphological classification, genetic diversity; SSR molecular markers; accessions

1. INTRODUCTION

Spinach (Spinacia oleracea L.), a member of the Chenopodiaceae family, is a globally cultivated vegetable valued for its broad adaptability, storability, long harvesting period, and versatile cultivation methods. Originating in pre-Persian regions [1,2], spinach was introduced to China via Nepal during the Tang Zhenguan era in the 7th century, and subsequently spread to Europe and the United States. Recognized as a plant with both medicinal and edible uses, spinach is consumed as a vegetable and utilized in traditional medicine. It was documented as “Persian grass” in Li Shizhen’s Compendium of Materia Medica [3]. From the Song and Yuan dynasties onward, as spinach cultivation expanded across China, it began to be recorded in local chronicles [4].

According to Records of Shanghai Vegetable Varieties [5], Chongming small spinach is an excellent local cultivar from the Chongming area of Shanghai, with a long history of cultivation in several towns including Xianghua, Hexing, and Hegangyan. Distinct from common commercial spinach varieties, Chongming small spinach is characterized by its short stature, rounded stems and leaves, thick mesophyll, and versatile culinary uses—suited both for stir-frying and cold dishes. It is praised for its fresh, sweet flavor, tender texture, and high nutritional value. This variety has gained popularity in the market for its superior taste and unique morphology, making it highly favored among consumers in China.

Spinach varieties are primarily classified based on seed morphology (round or spiny), leaf texture (smooth or wrinkled), color, leaf shape, and plant architecture [6]. For instance, Wu et al. [7] evaluated the botanical characteristics of 33 spinach accessions from diverse sources and classified them into two distinct groups through cluster analysis, identifying subgroups with pronounced morphological advantages to support future breeding efforts. Similarly, Yao et al. [8] assessed the morphological traits of 40 spinach accessions and categorized them into four groups using cluster analysis.

Simple sequence repeats (SSRs), or microsatellites, are short tandem repeats of 1–6 nucleotides widely employed as molecular markers. Their advantages include genome-wide coverage, robustness, high reproducibility, codominant inheritance, high polymorphism, transferability across species, and low technical and instrumental requirements [9–12]. Moreover, SSR genotyping is cost-effective and feasible for small-scale laboratories. These markers have been extensively used in fingerprinting, genetic diversity studies, population structure analysis, association mapping, and linkage mapping. For example, Khattak et al. [13,14] analyzed the genetic diversity of 33 hybrid spinach varieties using 13 SSR markers, grouping them into three clusters according to geographical origin. In another study, Kuwahara et al. [15] employed six SSR markers to evaluate 50 spinach accessions and revealed high genetic diversity, particularly among materials from West Asia (Afghanistan, Iran, Iraq, and Syria).

With the increasing commercialization of vegetable seeds, new domestic and international varieties possessing desirable commercial traits and high profitability are progressively replacing traditional local varieties. This trend has led to genetic homogenization in vegetable production and a decline in the cultivation of indigenous specialty varieties. Chongming small spinach, a high-quality local variety known for its excellent flavor and strong cold tolerance—especially as a winter-hardy type—has recently suffered from significant genetic degradation. Thus, there is an urgent need to collect, conserve, and characterize its germplasm resources. To date, no systematic study on the genetic diversity of the Chongming spinach population has been reported [16,17].

In this study, 42 spinach accessions were analyzed using a combination of morphological traits and SSR molecular markers to investigate the genetic relationships and diversity of Chongming small spinach. The objectives were to clarify the genetic structure of spinach germplasm from Chongming Island, support the conservation and utilization of these resources, and contribute to the genetic theory underlying spinach breeding. This research also provides a scientific basis for distinctness, uniformity, and stability (DUS) testing and variety protection of Chongming small spinach.

2. MATERIALS AND METHODS

2.1 Experimental material

A total of 42 spinach accessions from diverse geographical regions were used in this study, kindly provided by the Spinach Research Team of Shanghai Normal University, China. The collection comprised two accessions from the Netherlands, three from Russia, two from the United States, two from India, two from Turkey, one from Japan, one from Hong Kong (China), and 29 from various inland regions of China (Table S1). Among the Chinese accessions, six (B25, B26, B33, B34, B35, and B40) represent Chongming spinach, a local variety from Shanghai. These materials originate from different continents and have been cultivated under distinct environmental conditions (Figure 1). The experiment was conducted at the experimental base of Fengxian Campus, Shanghai Normal University, where approximately 30 seedlings per accession were sown using direct seeding in January 2020. Conventional field management practices were applied throughout the growing period. The plot design consisted of 3 m long rows with row spacing of 0.6 m and plant spacing of 0.25 m. The experimental site is characterized by an average annual temperature of 16°C, an annual sunshine duration of 1919.8 hours, and an annual rainfall of 1221.4 mm.

Figure 1 The distribution of 42 spinach accessions

2.2 Standards of phenotypic traits and investigation methods

Following the Description and Data Standard of Spinach Accessions [18], both qualitative and quantitative morphological evaluations were conducted. Ten qualitative phenotypic traits—including strain, straightness, leaf shape, leaf tip, leaf base, leaf fold, leaf gloss, leaf color, shank color, and cracking—were systematically investigated. In addition, six quantitative morphological indexes (plant height, plant width, leaf length, blade width, petiole length, and petiole width) were measured using a tape measure. At the three-leaf stage, ten individual plants per accession showing consistent growth were randomly selected for phenotypic assessment and measurement.

2.3 DNA extraction

At the three-leaf stage, leaf tissue samples were collected from three individual plants per variety and immediately flash-frozen in liquid nitrogen. Total genomic DNA was extracted using the CTAB method [19]. DNA concentration was measured both before and after dilution with a Thermo Scientific NanoDrop spectrophotometer. The DNA was then diluted to a working concentration of 100 ng/μL using ddH$_2$O and stored at −20°C for subsequent analyses.

2.4 Simple Sequence Repeats (SSR) analysis

The SSR primers used in this study were derived from previously published reports [20–22], and their sequences are listed in Table S2. All primers were synthesized by Shanghai Shenggong Biotechnology Co., Ltd. Polymerase chain reaction (PCR) was carried out in a total volume of 20 μL, consisting of 15 μL PCR Master Mix, 2 μL ddH$_2$O, 1 μL forward primer, 1 μL reverse primer, and 1 μL DNA template (100 ng/μL). Amplified products were separated via polyacrylamide gel electrophoresis (PAGE) following a previously described protocol [23].

2.5 Data analysis

The SSR amplification products were scored as 1 (present) or 0 (absent) according to the presence of clear and reproducible electrophoresis bands, resulting in a binary data matrix. Genetic diversity was evaluated using Popgene32 software [24] to determine the total number of alleles (Na), effective number of alleles (Ne), Nei's genetic diversity index (he), and Shannon's information index (I). A dendrogram was constructed with NTSYSPC 2.10 [25] based on the unweighted pair-group method with arithmetic mean (UPGMA). Pairwise genetic distances were calculated from Dice similarity coefficients using the formula: Distance =1−Similarity, followed by cluster analysis of the resulting distance matrix. All statistical analyses and data organization were performed using Microsoft Excel 2003. One-way ANOVA and additional cluster analyses were conducted with SPSS 26 (IBM).

3. RESULTS

3.1. Morphological characterization of 42 spinach accessions

Ten ordinal morphological traits were characterized across the 42 spinach accessions (Table S3). The assessed traits included: plant-type plant type (categorized as upright, semi-upright, or spreading), straightness (sagging, semi-sagging, or straight), leaf shape (sub-circular, oval, halberd-shaped, or lanceolate), and leaf tip morphology (round, pointed, or sharp-pointed). Additionally, leaf gloss (presence/absence), types of leaf folding, variations in leaf color, shank color polymorphism, and patterns of leaf cracking were also characterized.

3.1 Six quantitative traits

Morphological traits of the spinach accessions were measured using standardized tools and expressed in metric units. Analysis of variance revealed significant differences ($p<0.01$) among accessions for all traits evaluated (Table 1). Plant height ranged from 3.2 cm (B37) to 21.4 cm (B26), and plant width varied from 10.7 cm (B37) to 37.47 cm (B26), indicating that B37 was the smallest accession and B26 the largest in terms of overall plant size. Similarly, leaf dimensions also exhibited considerable variation: B37 displayed minimal values, with leaf length of 4.37 cm, leaf width of 4.83 cm, petiole length of 2.37 cm, and petiole width of 0.1 cm. In contrast, B26 showed the largest leaf traits, measuring 12.5 cm in leaf length, 8.87 cm in leaf width, 9.4 cm in petiole length, and 0.56 cm in petiole width.

Table 1 Determination results of morphological characterization of spinach

Serial number	**Plant height (cm)**	**Plant width (cm)**	**Leaf length (cm)**	**Leaf width (cm)**	**Petiole length (cm)**	**Petiole width (cm)**
B1	7.93±1.97	25.67±4.56	9.83±1.31	7.23±2.08	5.1±1.06	0.47±0.06
B2	10.57±2.28	25.47±3.7	14.2±3.11	8.73±3.19	4.13±0.5	0.43±0.06
B3	4.93±1.27	17.77±1.7	6.17±0.91	4.3±0.26	4.82±0.9	0.27±0.06
B4	20.5±4.33	37.53±7.43	10.17±1.25	9.83±2.57	11.52±2.79	0.43±0.21
B5	14.17±2.14	27.17±6.54	12.47±1.83	7.63±0.89	6.66±1.47	0.37±0.05
B6	11.87±1.5	24.6±7.01	9.3±2.35	5.8±0.87	9.45±1.01	0.3±0.03
B7	12.47±4.05	27.1±4.4	11.43±1.52	6.03±1.21	7.93±1.6	0.33±0.12
B8	7.5±1.54	23.87±2.28	8.57±0.45	5.87±1.79	6.37±1.36	0.3±0.05
B9	8±2.84	34.83±6.29	11.13±1.3	8.33±1.19	6.67±0.7	0.37±0.06
B10	5.82±0.84	15. 87±3.37	6.81±1	5. 37±0.85	3.38±0.15	0.29±0.06
B11	15.57±6.21	28.37±5.25	12.77±1.36	7.7±0.56	7.37±1.07	0.5±0.1
B12	11.93±0.85	18.4±2.49	8.87±1.45	6.23±1.32	4.87±1.25	0.37±0.06
B13	13.87±1.58	33.47±12.88	21.87±11.67	8.1±1.99	9.33±2.08	0.43±0.15

B14	7.57±2.12	12.8±0.6	5.9±1.08	13.33±1.62	3.97±1.1	0.3±0.05
B15	18.9±2.46	33.4±4.19	19.07±1.91	12.53±0.9	8.2±2.94	0.53±0.06
B16	12.6±1.44	29.17±3.87	11.6±0.69	7.87±1.06	9.43±1.65	0.47±0.06
B17	14.07±0.12	36.57±4.73	12.77±1.1	10.67±1.89	9.83±1.88	0.43±0.06
B18	3.59±1.13	10.74±2.26	4.25±0.42	4.16±0.31	2.18±0.53	0.21±0.03
B19	15.13±3.36	31.83±8.54	13.4±3.11	8.07±1.03	10.37±1.46	0.5±0.2
B20	12.1±0.17	30.33±10.87	12.07±3.27	6.97±1.03	8.27±2.37	0.43±0.06
B21	10.8±1.51	17.8±0.62	8.13±0.47	5.2±0.9	4.57±0.57	0.37±0.06
B22	13.17±1.53	21.17±5.11	9.47±2.55	7.6±1.95	6.65±2.1	0.37±0.06
B23	8.77±1.37	22.77±4.81	8.53±1.63	5.97±0.78	5.8±0.62	0.4±0.11
B24	9.03±2.38	26.8±5.72	9.97±1.6	6.62±1.56	6.57±1.37	0.4±0.12
B25	9.83±1.51	18.22±0.62	8.41±0.47	5.19±0.9	5.38±0.57	0.38±0.06
B26	21.4±4.13	37.47±8.95	12.5±2.07	8.87±0.74	9.4±1.32	0.56±0.05
B27	8.11±1.12	12.8±2.51	5.3±0.51	4.97±0.13	4.13±0.17	0.36±0.04
B28	14.6±2.76	25.93±4.36	10.27±1.36	6.91±0.45	8.37±1.47	0.39±0.03
B29	5.73±0.84	16.17±3.37	6.43±1	4.77±0.85	2.83±0.15	0.27±0.06
B30	5.8±0.67	10.6±2.51	4.6±0.42	4.93±0.17	2.67±0.14	0.34±0.04
B31	12.6±1.44	29.17±3.87	11.6±0.69	7.87±1.06	9.43±1.65	0.47±0.06
B32	13.41±1.5	22.59±7.01	8.94±2.35	5.78±0.87	8.83±1.01	0.33±0.02
B33	11.61±0.85	17.95±2.49	9.36±1.45	6.32±1.32	5. 97 ±1.25	0.37±0.06
B34	15.13±3.36	31.83±6.47	13.4±3.11	8.07±1.03	10.37±1.46	0.5±0.2
B35	5.3±0.47	8.8±1.31	4.1±0.13	4.2±0.16	2.69±0.14	0.32±0.02
B36	8±1.17	34.83±8.54	11.13±2.57	8.33±0.93	6.67±0.32	0.37±0.05
B37	3.2±1.13	10.7±2.26	4.73±0.42	4.83±0.31	2.37±0.13	0.1±0
B38	7.2±1.54	11.6±2.42	5.2±0.87	4.6±0.51	2.36±0.16	0.26±0.04
B39	12.62±1.44	27.17±3.87	9.65±0.69	8.64±1.06	9.18±1.65	0.39±0.06
B40	14.27±0.12	34.57±4.73	11.77±1.1	9.63±1.89	8. 93±1.88	0.46±0.06
B41	4.33±1.13	12.24±2.26	5. 23 ±0.42	3. 43±0.31	2.44±0.53	0.21±0
B42	15.13±3.36	31.83±6.72	13.4±3.11	8.07±1.03	10.37±1.46	0.38±0.2
Mix	3.2	8.8	4.1	3.4	2.2	0.1
Max	21.4	37.5	21.9	13.3	11.5	0.6
Mean±SE	11.2 ± 0.9	24.6 ± 1.5	9.8 ± 0.7	6.9 ± 0.4	6.5 ± 0.3	0.37 ± 0.02
F-value	24.7***	18.3***	15.9***	12.4***	22.1***	19.8***
p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

*** p<0.001; SE = standard error of the mean*

3.2. Morphological cluster analysis of spinach

Morphological cluster analysis was performed on 42 spinach accessions using six quantitative traits (Figure 2). The results showed that the accessions could be divided into two major groups (Group I and II) at a Euclidean distance of 25. Group I contained 26 accessions, while Group II comprised 16.

Group I was further subdivided into four distinct subgroups. Subgroup 1 consisted of four accessions (B9, B36, B17, and B40), which were characterized by a spreading plant type, semi-sagging leaves, flat leaf base, and glossy leaf surface. Subgroup 2 included nine accessions (B5, B11, B20, B39, B31, B16, B34, B42, and B19); these exhibited semi-sagging leaves with varying degrees of wrinkling, plant heights between 12–15 cm, and plant widths ranging from 27–31 cm. Subgroup 3 comprised nine accessions (B1, B2, B6, B7, B8, B23, B24, B28, and B32), featuring wrinkled, flat, or concave leaves that were yellow-green and shiny. Subgroup 4 contained four accessions (B4, B26, B13, and B15), all displaying a spreading growth habit along with smooth and glossy leaves (Table S3).

Group II was further subdivided into two subgroups. Subgroup 1 consisted of five accessions (B21, B25, B33, B12, and B22), characterized by a semi-upright plant type and light green, smooth, and glossy leaves. Subgroup 2 comprised 11 accessions (B3, B29, B10, B27, B38, B18, B41, B37, B30, B35, and B14), which exhibited distinct morphological traits compared to other groups. These accessions displayed a compact plant architecture, with plant heights ranging from 3.59 cm to 8.11 cm and plant widths between 8.81 cm and 12.24 cm. The leaves were predominantly oval or suborbicular in shape, dark green in color, glossy, and slightly wrinkled.

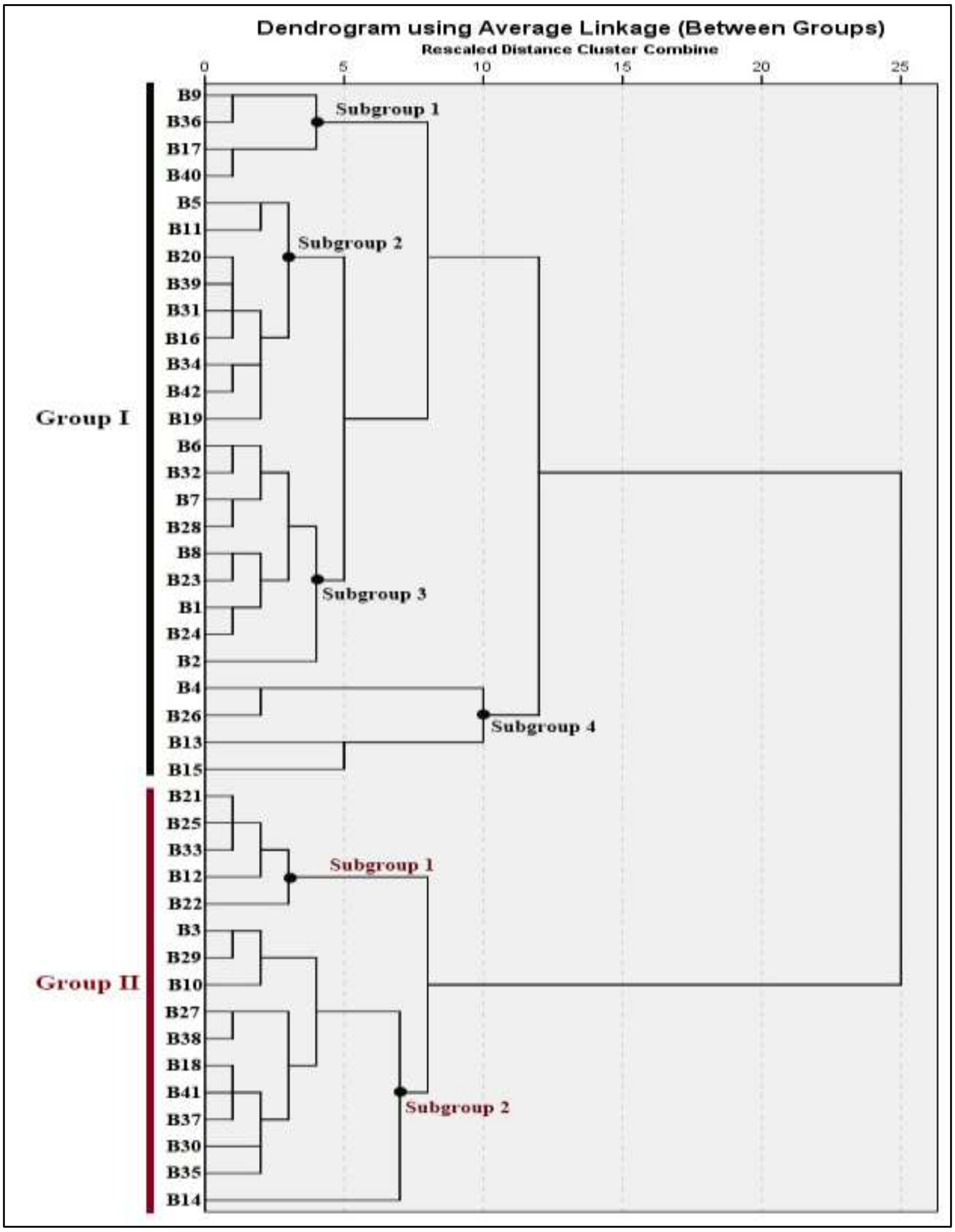

Figure 2 UPGMA dendrogram of 42 spinach accessions based on their morphological characteristics

3.3. SSR primer screening and polymorphism analysis

A total of 300 SSR primer pairs were screened for polymorphism across the 42 spinach accessions. Among these, 57 primer pairs produced clear amplification bands and exhibited high polymorphism, and were therefore selected for further analysis. An example amplification profile from primer 47 (chr04_3_31208653a) is presented in Figure 3. These 57 primers collectively amplified 198 alleles, of which 178 (89.9%) were polymorphic across the studied accessions. On average, each primer amplified 3.06 polymorphic loci. The number of alleles per locus ranged from 2 to 10, with a mean of 3.10 (95% CI: 2.85-3.35). The effective number of alleles varied from 1.40 to 3.89, averaging 3.09 (95% CI: 2.80-3.38). Nei's genetic diversity index (he) ranged from 0.24 to 0.74, with an average of 0.62 (95% CI: 0.58-0.66). Shannon's information index (I) varied from 0.9 to 1.52, averaging 1.18 (95% CI: 1.10-1.26).

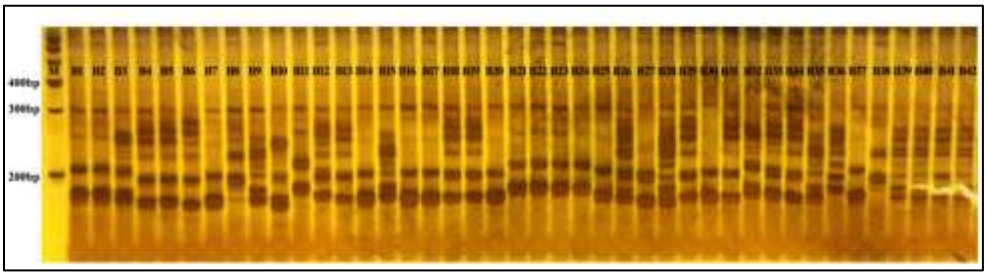

Figure 3 Amplification results of primer 47 representative SSR primers

3.4. Cluster analysis based on SSR

The genetic similarity coefficients (GS) among the 42 spinach accessions ranged from 0.30 to 0.75. Accessions B11, B23, B27, and B41 exhibited the highest genetic similarity, indicating close genetic relationships. An unweighted pair group method with arithmetic mean (UPGMA) cluster analysis was conducted based on the amplification profiles of the 57 polymorphic SSR primers. The resulting dendrogram illustrating the genetic relationships among the accessions is presented in Figure 4. At a genetic distance of 0.30, the germplasms were separated into two major clusters (Cluster I and II). Cluster I, consisting of 17 accessions, was further subdivided into three subclusters. Subcluster I comprised six accessions: one from the Netherlands (B1), one from Russia (B2), one from the United States (B3), two from Shandong Province, China (B29 and B36), and one from Shanghai, China (B37). Subcluster II included eight accessions: three from India (B4, B5), two from Russia (B10, B14), two from Songjiang, China (B30, B39), one from Hubei, China (B12), and one from the Netherlands (B20). Subcluster III contained three accessions: two from Fengxian, China (B27, B38), and one from Jiangsu, China (B41).

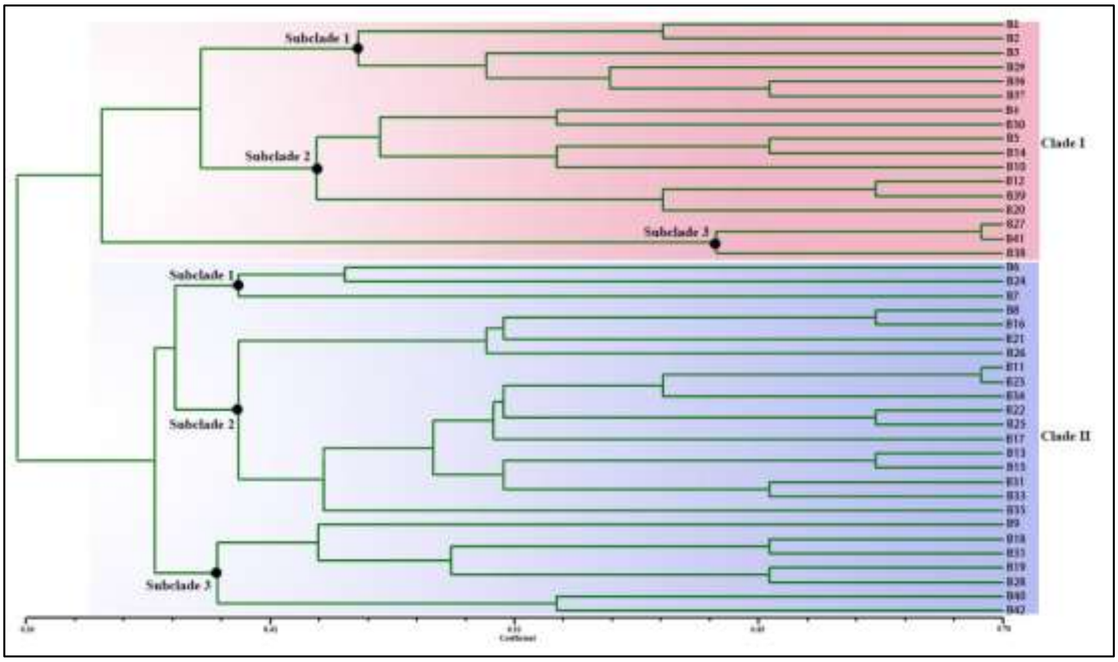

Figure 4 Clustering analysis of 42 spinach accessions based on SSR molecular marker.

Cluster II comprised 30 accessions and was further subdivided into three subclusters. Subcluster I contained three germplasms: one from Europe (B6), one from Hebei, China (B24), and one from Turkey (B7). Subcluster II included 15 germplasms, consisting of one from the United States (B8), one from Japan (B17), one from Hong Kong, China (B21), five from Chongming, China (B25, B26, B33, B34, B35), and seven from other regions of China. Subcluster III consisted of seven accessions, all originating from various regions of China (B9, B18, B19, B28, B31, B40, and B42).

3.5. Fingerprint

Out of the 57 primers evaluated, nine were selected for their ability to produce distinct polymorphic bands that clearly differentiated among the varieties (Tables 1 and 3). These core primers collectively revealed 68 polymorphic sites and 63 unique genotypic band combinations. A fingerprinting profile for the 42 spinach accessions was established based on these 63 unique band patterns (Table 4).

Specifically, primers 9 and 46 successfully distinguished accession B35 (Chongming spinach) and B26 from all other varieties. Primer 40 differentiated B35 and B40 from the remaining accessions. These primers are therefore recommended for use in variety identification of spinach germplasm. It should be noted that, due to the limited number of primers and accessions included in this study, the constructed fingerprint database is currently applicable only to the materials analyzed.

Table 2 Genetic diversity parameters

Locus	**na***	**ne***	**h***	**I***	**PIC**
Primer 9	12	9.73	2.15595	3.17245	0.977769117
Primer 15	2	1.26875	0.20525	0.35395	0.610827664
Primer 34	9	6.9717	1.39805	2.0445	0.947887797
Primer 40	8	6.32565	1.43085	2.1562	0.95018424
Primer 44	7	5.81885	1.347	1.99045	0.951686797
Primer 46	11	7.21535	1.1655	1.904	0.959258635
Primer 47	7	5.66075	1.28915	1.92885	0.938844926
Primer 52	7	5.1846	0.9722	1.43365	0.921201814
Primer 56	5	2.82455	0.4518	0.64415	0.897120181

Table 3 Basic information of 9 pairs of core primers

Primer number	Marker name	Polymorphic site	Number of unique genotypic band combinations
Primer 9	SO3	12	12
Primer 15	SO17	2	3
Primer 34	chr02_5_18463938a	9	9
Primer 40	chr03_3_9824683a	8	8
Primer 44	chr03_3_75066435	7	7
Primer 46	chr05_3_1063291a	11	9
Primer 47	chr04_3_31208653a	7	6
Primer 52	chr01_6_26485152a	7	6
Primer 56	chr04_3_5365603	5	3

Table 4 Fingerprinting of 42 spinach accessions constructed by 9 pairs of SSR primers

NO	Primer number and fingerprint number								
	9	15	34	40	44	46	47	52	56
B1	110011000100	11	011100100	11001101	0100110	11000111111	1100111	1111011	11100
B2	011000110011	11	111101110	11001101	1100110	11001110011	1101001	1110111	11110
B3	110011001001	11	010011100	11001101	0101011	11001110010	1110110	1100111	11100
B4	110001100010	11	111101110	01010111	1100111	11100110010	1100111	1001011	01100
B5	110000110010	11	111001110	10101001	0100110	11000110010	0001011	1111011	11110
B6	101000110010	11	010011100	11101100	1100110	11100110010	0001011	1101011	11110

B7	101010110010	11	111101110	11001111	0101011	11100110010	0101011	1100001	11100
B8	011000110010	11	111001110	11000110	1100111	11000110011	1101011	1101011	01100
B9	110011000101	11	111111110	11001101	1110110	11010111111	1101001	1100001	11110
B10	110011001001	11	011100100	01010111	1010000	11000110010	1110110	1110111	11100
B11	001000110010	11	111101110	10101001	1110000	11000110010	1100111	1100111	01100
B12	110000110010	11	010011100	11101100	1110110	11100110010	0001011	1001011	11110
B13	101000110010	11	111101110	11001111	1010000	11100110010	0101011	1111011	11100
B14	101010110010	11	111111110	11000110	1110000	11000110011	1101011	1101011	01100
B15	011000110010	11	010100111	01001001	0100110	11010111111	0001011	1100001	11110
B16	110011000101	11	111101110	11001101	1100110	11000110010	0101011	1110111	11100
B17	110011001001	10	111001110	01001001	0101011	11000110010	1101011	1100111	01100
B18	110000110010	11	111101110	11001101	1110110	11000110010	0001011	1100111	01100
B19	110001100010	01	011100100	11001101	1110110	11000111111	1110110	1110111	11100
B20	110000110010	11	010101100	01010111	1010000	11001110011	1100111	1100111	01100
B21	101000110010	11	010011100	10101001	1110000	11001110010	1101001	1001011	11110
B22	101010110010	10	111101110	11101100	0100110	11100110010	1110110	1111011	11100
B23	011000110010	10	111101110	11001111	1100110	11000110011	1100111	1101011	01100
B24	110011000101	11	111001110	11000110	0101011	11010111111	0001011	1100001	11110
B25	110011001001	11	111111110	01001001	1100111	11000110010	0101011	1111011	11100
B26	001000110010	11	011100100	11001101	1110110	11000110010	1101011	1101011	01100
B27	111000110010	11	010101100	11001101	1010000	11100110010	1101001	1100001	11110
B28	110001100010	11	010011100	01010111	1110000	11100110010	1110110	1110111	11100

B29	110000110010	11	111101110	10101001	0100110	11000110011	1100111	1100111	01100
B30	101000110010	11	111001110	11101100	1100110	11010111111	0001011	1001011	11110
B31	101010110010	11	111111110	11001111	0100110	11000110010	1100111	1111011	11100
B32	011000110010	10	011100100	11000110	1100110	11000110010	1101001	1101011	01100
B33	110011001001	01	010101100	01001001	0101011	11100110010	1110110	1100001	01100
B34	110001100010	11	010011100	11001101	1100111	11000110010	1100111	1110111	11110
B35	110001010010	10	111111110	01111000	1100111	10011000100	1101001	1001011	11110
B36	110001010010	10	010101101	01010111	1010000	10011000100	0001011	1001011	11110
B37	101010110010	10	010100111	01111000	1110000	11100110010	0101011	1111011	11100
B38	011000110010	11	010101100	11101100	0101011	11000110011	1100111	1101011	01100
B39	110011000101	11	010011100	11001111	1100111	11010111111	1101001	1100001	11110
B40	110011001001	01	111101110	11000110	1110110	11000110010	1110110	1110111	11100
B41	001000110010	11	010101101	01001001	1010000	11000110010	1100111	1100111	01100
B42	111000110010	11	010100111	11001101	1110000	11100110010	0001011	1001011	11110

4. DISCUSSION

4.1. Genetic diversity

Spinach is believed to have originated in ancient Persia and began spreading globally around the 11th century [23]. Geographic isolation, along with natural and artificial selection under diverse climates, has contributed to its considerable genetic diversity. In recent years, SSR markers have become a widely adopted tool for assessing genetic diversity and identifying spinach varieties. For example, Zhao et al. [24] used PAGE to develop fingerprints and evaluate diversity among 33 accessions, screening 27 polymorphic primer pairs that amplified 109 loci, with an average of 4 loci per primer. Goldberg et al. [25] applied 85 markers to 48 global varieties, detecting 389 alleles (89% polymorphic), and demonstrated that SSR markers effectively evaluate genetic structure and group germplasm by geographical origin. Wang [26] reported moderate polymorphism (PIC=0.43) and relatively low genetic diversity among 43 Chinese varieties using 41 SSR primers.

In this study, 57 SSR primers were used to analyze 42 global accessions. The genetic similarity coefficients ranged from 0.30 to 0.75, and the polymorphic locus percentage was 89.90%, consistent with Goldberg et al. [25], confirming the utility of our markers. Notably, while SSR markers revealed clear genetic groupings correlated with geography, morphological clustering based on six quantitative traits showed less distinct subgroup separations, with varieties from different genetic clusters overlapping in phenotypic space (Figure 1–3). This discrepancy highlights the complementary value of combining morphological and molecular approaches. Morphological traits, while reflecting genetic background, are also strongly influenced by the environment. For instance, Chongming spinach exhibited plasticity in plant architecture—upright under facility cultivation but spreading in open fields—demonstrating how environmental factors like light and temperature alter phenotypic expression [27,28]. Thus, morphological traits should serve as a supplementary tool in diversity studies, with SSR markers providing a more stable, genetically grounded classification.

4.2. Genetic relationship and distinctiveness of Chongming spinach

Although the exact migration routes of spinach from Iran remain unclear, it likely spread along two paths: to South/East Asia and to Africa/Europe/Americas, leading to the divergence between Asian and Western varieties [29–32]. In this study, SSR analysis separated the accessions into Clade I (northern China, Russia, Europe, America) and Clade II (Shanghai, southern China, East Asia) at a genetic distance of 0.30 (Figure 3). Chongming spinach accessions (B26, B35, B40) grouped within Clade II, indicating shared genetic ancestry with southern Chinese and East Asian materials and supporting their regional origin.

Despite shared genetic background, morphological variation among Chongming accessions and other Shanghai varieties was evident, likely resulting from environmental adaptations, natural hybridization, and local cultivation practices. This phenotypic divergence underlines the influence of non-genetic factors on morphology. Importantly, the genetic distinctiveness of Chongming spinach was robustly captured by SSR markers. Using three primers (9, 40, 46), we distinguished B26, B35, and B40 from all other accessions (Table 4), confirming their unique and identifiable genotype. Limited gene flow and significant genetic drift have further narrowed the genetic diversity of Chongming spinach [33], enhancing its population-specific characteristics.

The combined morphological and molecular evidence clarifies the origin and uniqueness of Chongming spinach: while environmentally mediated phenotypic plasticity explains the within-group morphological variation, SSR markers consistently reflect its genetic identity and southern Chinese origin. As market demands drive increased varietal numbers and phenotypic convergence [34], molecular fingerprints provide a reliable tool for variety identification [35–38]. The core primer set developed here offers an efficient method for conserving and utilizing Chongming spinach germplasm.

5. CONCLUSIONS

In this study, the genetic diversity of 42 spinach accessions was analyzed, and the DNA fingerprint database was constructed to clarify the genetic relationship between varieties to provide a basis for the protection of intellectual property rights of new varieties, variety identification, and innovative breeding of Chongming small spinach, as well as to increase the number of fingerprints for spinach, enrich the number of SSR markers, and improve the spinach DNA fingerprint database.

Supplementary Materials: Table S1: Spinach material number and origin; Table S2: List of 57 pairs of SSR primers; Table S3: Determination results of morphological indexes of spinach.

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