

Article

Optimization and Application of SSR Molecular Markers in Pepper Variety Identification

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Highlights

What are the main findings?

- We developed an optimized SSR core primer set consisting of 26 pairs with an average PIC of 0.49 and a discrimination efficiency of 99.975% across 240 pepper varieties.
- We constructed a DNA fingerprint database for 240 pepper varieties and identified six genetic groups that showed good correlation with phenotypic traits.

What are the implications of the main finding?

- The system offers a reliable and high-throughput tool for pepper authenticity and purity testing, parentage analysis, and DUS similar variety preselection.
- It strengthens new variety rights protection and supports the standardized management of the pepper seed industry.

Abstract

Pepper breeding in China faces challenges including narrow genetic backgrounds, variety homogenization, and authenticity issues in the seed market. The original SSR-based variety identification system (NY/T 2475-2013) has limitations such as low polymorphism and uneven marker distribution, failing to meet current efficiency requirements. In this study, 240 pepper varieties were used. Fluorescent capillary electrophoresis was employed to analyze issues in the original system. A total of 150 SSR primer pairs was screened using eight phenotypically distinct varieties, followed by rescreening with 96 varieties from different origins. After marker optimization, a new identification system was established, validated using 144 varieties, and a DNA fingerprint database was constructed. Phenotypic experiments were conducted to evaluate applicability. The new system comprises 26 core primer pairs: 12 newly selected and 14 retained from the original standard. These primers are evenly distributed across chromosomes, with annealing temperatures optimized to 57 °C and grouped into seven multiplex panels. Across 240 pepper varieties, the 26 primer pairs detected 153 allelic variants and 293 genotypes. Average polymorphism information content (PIC) increased from 0.43 to 0.49 compared to the original system, and the discrimination efficiency reached 99.975% among 28,680 pairwise variety combinations. Phylogenetic and population structure analyses divided the 240 pepper varieties



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into six groups. Varieties with molecular genetic distance approaching zero showed high phenotypic similarity. The newly developed SSR-based system demonstrated excellent performance and strong applicability. It can be used for authenticity and purity identification, parentage testing, and auxiliary selection in DUS testing, supporting new variety rights protection and providing technical support for standardized management of the pepper seed industry.

Keywords: pepper; SSR; variety identification; genetic diversity

1. Introduction

Pepper (*Capsicum* L.), originating from Central and South America, belongs to the Solanaceae family and is an important vegetable and spice crop cultivated worldwide [1,2]. According to FAO data, the global pepper planting area reached 3.68 million hectares in 2021, with fresh pepper production of 36.29 million tons. China is the world's largest pepper producer, with 760,000 hectares and 16.75 million tons [3]. Currently, 42 *Capsicum* species have been reported, with five cultivated species: *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum*, and *C. pubescens* [4].

Currently, the genetic diversity of pepper varieties in China is lower than that in some other countries, and artificial selection has further reduced the genetic diversity of breeding lines [5]. Issues such as low-level repetitive breeding, severe variety homogenization and fragmentation, narrow genetic backgrounds, and difficulties in variety protection pose higher demands on pepper variety identification technology [6]. Furthermore, by the end of 2023, the number of pepper variety protection applications had reached 1756. The annual increase in applications presents new challenges for efficient variety identification. In the market, issues like “same variety with different names” and “same name for different varieties” significantly harm breeders' rights and pose risks to the healthy development of the pepper industry. Therefore, constructing high-quality, efficient molecular identification standard systems and DNA fingerprint databases for pepper variety protection is crucial for addressing these issues and supporting variety registration, management, and rights protection.

Traditional morphological variety identification relies on observing and describing plant characteristics to determine taxonomic status. However, this method is time-consuming, requires highly skilled personnel, and is susceptible to environmental conditions and human error [7]. Molecular marker technology directly reflects DNA-level differences, effectively overcoming the limitations of morphological identification [8]. Simple sequence repeats (SSRs) are tandem repeat DNA sequences consisting of one to six nucleotide units, widely distributed in prokaryotic and eukaryotic genomes. SSR markers offer co-dominant inheritance, multiple alleles, broad genome coverage, high abundance, and high reproducibility [9,10]. They are widely used in genetic map construction, genetic diversity and population structure analysis, and genome-wide association studies [11–13]. The International Union for the Protection of New Varieties of Plants (UPOV) considers molecular markers a powerful tool for distinguishing varieties and lines (including hybrid parents and inbred lines), recommending them as a preferred marker type for plant variety identification and database construction [14].

The completion of whole-genome sequences and assemblies for several pepper varieties—including Zunla-1, CM334, and CA59 [2,15,16]—has greatly facilitated the development of SSR markers, which are now widely applied in genetic diversity analyses and variety identification. For instance, Zhong et al. [17] employed 33 SSR markers to assess

147 *C. frutescens* accessions from 25 countries, revealing seven clusters that largely mirror geographic origins, thereby underscoring the utility of SSR markers for tracing population structure at a global scale. Similarly, Guo et al. [5] combined 26 SSR markers with one InDel to compare 94 local landraces and 85 modern breeding lines from China, and their clustering patterns clearly separated the two groups, suggesting that selection during breeding has left a detectable genetic imprint. Extending this approach to gene-based markers, Li et al. [18] designed 17 SSRs from coding regions and, through Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis, classified 169 accessions into seven categories that align well with the basic taxonomic divisions of *Capsicum*, reinforcing the consistency between marker-derived groupings and conventional species boundaries. On a much larger scale, Gu et al. [19] screened 1904 pepper accessions with 29 SSRs, identifying two major groups distinguished by fruit morphology and geographical provenance, which highlights the power of SSR panels for resolving broad-scale diversity patterns even in massive germplasm collections. Beyond genetic clustering, the choice of marker system itself can influence resolution; Chen et al. [20] directly compared Sequence-Related Amplified Polymorphism (SRAP) and SSR markers across eight pepper accessions and found that SSRs exhibited higher polymorphism and greater discriminatory ability, suggesting that SSR-based approaches may offer superior efficiency for routine fingerprinting and diversity surveys. Collectively, these studies not only demonstrate the versatility of SSR markers across different population sizes and taxonomic levels, but also show that the resulting classifications consistently capture meaningful biological and geographical signals, providing a robust framework for pepper germplasm management and breeding.

China's original pepper SSR variety identification standard (NY/T 2475-2013) was released on 13 December 2013 [21]. Before 2013, SSR marker development in pepper faced challenges due to incomplete whole-genome sequences and assemblies, unclear primer chromosomal locations, and insufficient primer screening and evaluation. Furthermore, the identification system based on polyacrylamide gel electrophoresis (PAGE) suffered from poor accuracy in result interpretation. Since 2014, whole-genome sequencing of several *C. annuum* (CM334, Zunla-1, CA59, Dempsey, Takanotsume) [2,14,15,22,23], *C. chinense* (PI159236), and *C. baccatum* (PBC81) [24] varieties have been completed, facilitating the construction of efficient and accurate SSR marker identification systems. Therefore, it is necessary to enhance the screening and evaluation of standard pepper primers, clarify core primer marker information, optimize the core primers system, and develop a high-sensitivity, accurate, high-throughput SSR identification system based on fluorescent capillary electrophoresis platforms.

This study evaluates the core primers of the original Chinese pepper SSR variety identification standard. Following the “General Principles for Plant Variety Identification Using DNA Molecular Marker Methods” (2016) (NY/T 2594-2016) [24], it aims to optimize and select a set of SSR core primers with stable amplification, clear peak patterns, high discrimination, and high polymorphism. This set will be used to establish a pepper variety molecular identification system and DNA fingerprint database, providing technical support for rapid authenticity identification, assisting in the selection of candidate varieties for DUS testing (Distinctness, Uniformity and Stability), and helping to regulate the pepper seed market.

2. Materials and Methods

2.1. Plant Materials

A total of 240 pepper varieties were obtained from the National Plant Variety Conservation Center, Ministry of Agriculture and Rural Affairs, including varieties from 24 provinces

in China, as well as from countries such as the United States, Japan, the Netherlands, and Switzerland (Table S1).

2.2. SSR Primers

A collection of 150 pepper SSR primer pairs with known chromosomal locations was gathered from the relevant literature (Table S2) [17,18,25–28]. After preliminary screening via conventional capillary electrophoresis, primers that exhibited clear, stable, and highly polymorphic amplification products were selected. These selected primers were then labeled at the 5' end of the forward primer with one of four fluorescent dyes (FAM, ROX, TAMRA, HEX) based on the expected amplicon size range. Regular and fluorescent primers were synthesized by Beijing Tsingke Biotechnology Co., Ltd. (Beijing, China).

2.3. Genomic DNA Extraction

Young leaves from 30 seedlings per variety were pooled and ground in liquid nitrogen. Genomic DNA was extracted using a modified CTAB method [29]. DNA concentration and purity (OD260/OD280) were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA stock solutions were diluted to 20 ng/μL and stored at −20 °C.

2.4. PCR Amplification

A 20 μL PCR reaction mixture was used, containing 0.5 μL each of forward and reverse SSR primers (10 μmol·L^{−1}), 2 μL DNA template (20 ng/μL), 10 μL 2× Taq Plus Master Mix, and 7 μL double-distilled water. The PCR program was as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 45 s; and a final extension at 72 °C for 10 min, held at 4 °C.

2.5. BLAST Alignment

Primers from the original standard (NY/T 2475-2013) were used for PCR amplification, followed by cloning and sequencing. Obtained sequences were aligned with the pepper reference genome [30] via NCBI BLAST (<https://www.ncbi.nlm.nih.gov/>) to determine their chromosomal locations.

2.6. Amplification Product Detection

Regular PCR products were diluted and analyzed using a Qsep400™ automated nucleic acid protein analysis system with 96-well plates (Bioptic Inc., New Taipei City, Taiwan, China). Amplified products from fluorescently labeled primers were diluted and pooled. One microliter of the mixed dilution was added to a deep-well plate containing 0.1 μL LIZ500 size standard and 8.9 μL deionized formamide. After denaturation at 95 °C for 5 min and cooling, fragment analysis was performed on an ABI3730 genetic analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Phenotypic Data Collection

Two variety pairs with zero allelic differences, four pairs with one allelic difference, and five candidate varieties provided by applicants were selected for field trials. Thirty-four phenotypic traits were recorded according to the agricultural industry standard “Guidelines for the Conduct of Tests for Distinctness, Uniformity, and Stability–Pepper (*Capsicum annuum* L.)” (NY/T 2234-2012).

2.8. Data Analysis

Allelic fragment size data were recorded. Homozygous loci were recorded as X/X, and heterozygous loci as X/Y (e.g., 201/201, 201/205). Power Marker V3.25 (North Carolina

State University, Raleigh, NC, USA) [31] was used to calculate major allele frequency, number of genotypes, polymorphism information content (PIC), and Nei's Genetic Distance (Nei 1983) [32] among varieties. GenAEx 6.5 (Australian National University, Canberra, ACT, Australia) [33] was used to calculate genetic diversity parameters such as number of alleles (Na), effective number of alleles (Ne), observed heterozygosity (Ho), and expected heterozygosity (He). MEGA 11 (Temple University, Philadelphia, PA, USA) [34] and iTOL v6 (EMBL Heidelberg, Heidelberg, Germany) [35] were used for phylogenetic tree construction. Structure 2.3.4 (Stanford University, Stanford, CA, USA) [36] was used to analyze population structure, with K ranging from 1 to 10, a burn-in period of 5000, MCMC iterations of 50,000, and 20 runs per K value. The optimal K was determined based on the maximum likelihood value.

NTSYS-pc 2.11 software (Exeter Software, Setauket, NY, USA) was used to calculate the phenotypic similarity coefficient matrix based on morphological traits, followed by UPGMA cluster analysis.

3. Results

3.1. Analysis of the Original Identification System

3.1.1. Allelic Variation Frequency and Polymorphism of Primers

Using the 22 core primer pairs from the original standard, 115 alleles and 223 genotypes were detected across 240 pepper varieties. The major allele frequency ranged from 0.22 (HPMS1-5) to 0.90 (ES133), averaging 0.64. PIC values ranged from 0.18 (ES133) to 0.86 (HPMS1-5), averaging 0.43 (Table 1). According to PIC criteria ($PIC < 0.25$: low polymorphism; $0.25 \leq PIC < 0.5$: moderate; $PIC \geq 0.5$: high), the original core primers included three low-polymorphism primers, 12 moderate-polymorphism primers, and seven high-polymorphism primers. Among low-polymorphism primers, ES133 had only three alleles and the lowest PIC (0.18), with a major allele frequency of 0.90. ES201 had four alleles, an effective allele number of 1.22, a PIC of 0.19, and a major allele frequency of 0.89 (Table 1). This indicates poor discriminative ability for these two primers across the 240 pepper varieties. Optimization of the original system should consider replacing such primers to improve overall polymorphism.

Table 1. Genetic diversity parameters of the 22 core SSR primer pairs from the original standard (NY/T 2475-2013) across 240 pepper varieties.

Primers	Number of Genotypes	Number of Observed Alleles	Number of Effective Alleles	Major Allele Frequency	Observed Heterozygosity	Expected Heterozygosity	Polymorphism Information Content
EPMS-923	8	6	2.34	0.47	0.34	0.57	0.52
EPMS697	4	3	1.94	0.58	0.32	0.48	0.41
EPMS712	5	3	2.02	0.51	0.22	0.51	0.39
ES105	6	6	1.42	0.83	0.13	0.29	0.28
ES110	6	4	1.38	0.83	0.20	0.27	0.26
ES120	21	9	3.75	0.33	0.38	0.73	0.70
ES133	5	3	1.22	0.90	0.12	0.18	0.18
ES167	9	5	1.87	0.69	0.34	0.47	0.45
ES201	7	4	1.22	0.89	0.13	0.18	0.19
ES212	5	4	1.33	0.86	0.15	0.25	0.22
ES285	5	3	1.73	0.70	0.27	0.42	0.34
ES292	7	4	1.32	0.85	0.12	0.24	0.26
ES297	3	3	1.70	0.71	0.22	0.41	0.33
ES321	10	5	1.78	0.71	0.32	0.44	0.44
ES330	8	4	2.73	0.47	0.41	0.63	0.58
ES363	11	6	2.80	0.46	0.29	0.64	0.60
ES395	10	6	2.73	0.49	0.35	0.63	0.58

Table 1. Cont.

Primers	Number of Genotypes	Number of Observed Alleles	Number of Effective Alleles	Major Allele Frequency	Observed Heterozygosity	Expected Heterozygosity	Polymorphism Information Content
ES417	9	5	1.47	0.79	0.15	0.32	0.34
ES64	8	7	1.94	0.62	0.32	0.48	0.41
HPMS1-106	6	3	2.01	0.56	0.28	0.50	0.46
HPMS1-214	14	7	2.70	0.54	0.34	0.63	0.58
HPMS1-5	56	15	7.05	0.22	0.61	0.86	0.86
Total	223	115	48.42	\	\	\	\
mean Average	\	5.23	2.20	0.64	0.27	0.46	0.43

3.1.2. Analysis of Allelic Variation at Primer Loci

Comparison between the allelic variants specified in the original standard and those detected in the 240 pepper varieties revealed that some specified variants were absent, while new variants were detected. For example, primer EPMS-923 did not detect the specified allele (342 bp) but detected three new alleles (253, 254, 308) along with three specified ones (274, 287, 306). Primer ES120 detected four new alleles (154, 164, 166, 167) in addition to five specified ones (155, 157, 159, 163, 165) (Table 2). This indicates that the SSR locus information in the original standard deviates from the actual composition of varieties bred in recent years, necessitating updates. Furthermore, using standard samples for variety protection as references ensures greater authenticity and representativeness compared to collecting arbitrary varieties.

Table 2. Allelic variation ranges of the 22 core SSR primer pairs from the original standard (NY/T 2475-2013) among 240 pepper varieties.

Primer	Allelic Variation (bp)	Primer	Allelic Variation (bp)
EPMS-923	253, 254, 274, 287, 306, 308	ES292	121, 125, 132, 135
EPMS697	112, 115, 118	ES297	110, 116, 122
EPMS712	137, 140, 143	ES321	106, 116, 120, 124, 148
ES105	121, 139, 149, 150, 151, 154	ES330	97, 109, 115, 121
ES110	138, 144, 147, 150	ES363	87, 89, 90, 91, 106, 107
ES120	154, 155, 157, 159, 163, 164, 165, 166, 167	ES395	84, 87, 93, 102, 103, 105
ES133	150, 153, 156	ES417	73, 76, 82, 88, 93
ES167	237, 238, 243, 249, 255	ES64	152, 155, 156, 160, 164, 166, 168
ES201	136, 139, 142, 145	HPMS1-106	154, 155, 160
ES212	140, 142, 143, 145	HPMS1-214	82, 88, 91, 94, 97, 100, 101
ES285	118, 119, 121	HPMS1-5	289, 291, 294, 299, 301, 303, 305, 307, 309, 311, 313, 315, 317, 318, 320

3.1.3. Determination of Chromosomal Locations

Following amplification and sequencing, BLAST alignment clarified the chromosomal locations of 19 markers from the original standard (Figure 1). Chromosomes 2 and 3 each had four SSR loci, with ES201 and EPMS697 closely located on chromosome 2. ES417, ES285, and ES212 were on chromosome 1. Chromosomes 7 and 9 had two loci each, and chromosomes 4, 8, 10, and 11 had one locus each. No loci were distributed on chromosomes 5 and 12. HPMS1-5, HPMS1-214, and HPMS1-106 could not be aligned to the published pepper genome. The literature review indicated that HPMS1-5 and HPMS1-214 were located on linkage groups 6 and 1, respectively, of the “SUN” genetic map developed in 2001 [37], while HPMS1-106 had not been successfully mapped. Failure to map these three loci to chromosomes likely results from the relevant genomic fragments not being assembled into the current pepper genome. These results show uneven distribution of SSR loci from the original standard across pepper chromosomes, which may affect the discriminatory efficiency of the identification system.

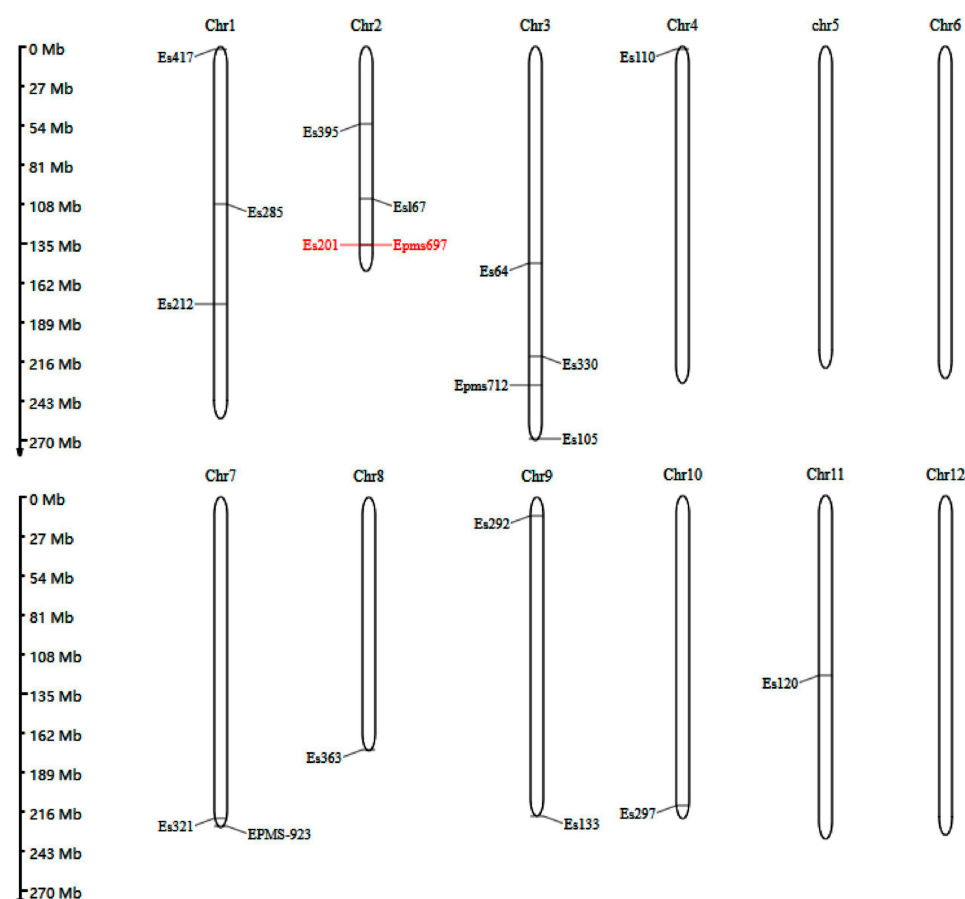


Figure 1. Chromosomal positions of 19 SSR markers (NY/T 2475-2013) in *Capsicum annuum* L. (NCBI:txid4072). HPMS1-5, HPMS1-214, and HPMS1-106 are unmapped.

3.2. Optimization and Application of Core Primers

3.2.1. Annealing Temperature Optimization

Core primers in the original standard had eight different annealing temperatures (52 °C, 53 °C, 54 °C, 55 °C, 56 °C, 58 °C, 60 °C, 65 °C), hindering detection efficiency. Through gradient experiments, the annealing temperature for all primers was adjusted to 57 °C. Amplification efficiency and results at 57 °C were comparable or superior to those at original temperatures when detected via fluorescent capillary electrophoresis (Figure 2), making 57 °C suitable for PCR amplification of these primers.

3.2.2. Screening and Replacement of Core Primers

Based on polymorphism analysis and chromosomal localization of the original core primers, primers were removed, replaced, or added. Primers with redundant distribution on the same chromosome and low polymorphism (e.g., ES212 on Chr1, EPMS697 and ES201 on Chr2, ES105 and EPMS712 on Chr3) were removed. Primers with unclear linkage (e.g., HPMS1-106) were removed. Primers with low polymorphism on chromosomes (e.g., ES133 and ES292 on Chr9) were replaced. New core primers were added to chromosomes lacking loci or possessing fewer than two marker loci.

Eight pepper varieties with large differences in botanical traits were selected for preliminary screening (Table 3). PCR amplification of these eight varieties using 150 primer pairs, followed by ordinary capillary electrophoresis, led to the preliminary selection of 36 primer pairs with stable amplification and high polymorphism. These 36 pairs were used for fluorescent PCR amplification of 96 pepper varieties. Fragment analysis on an ABI3730xl genetic analyzer, considering amplification stability, ease of peak reading, high

polymorphism, and even chromosomal distribution, resulted in the selection of 12 candidate primer pairs as core primers for the new standard. These were combined with 14 retained from the original standard, totaling 26 primer pairs, to establish the new pepper variety identification system (Table S3).

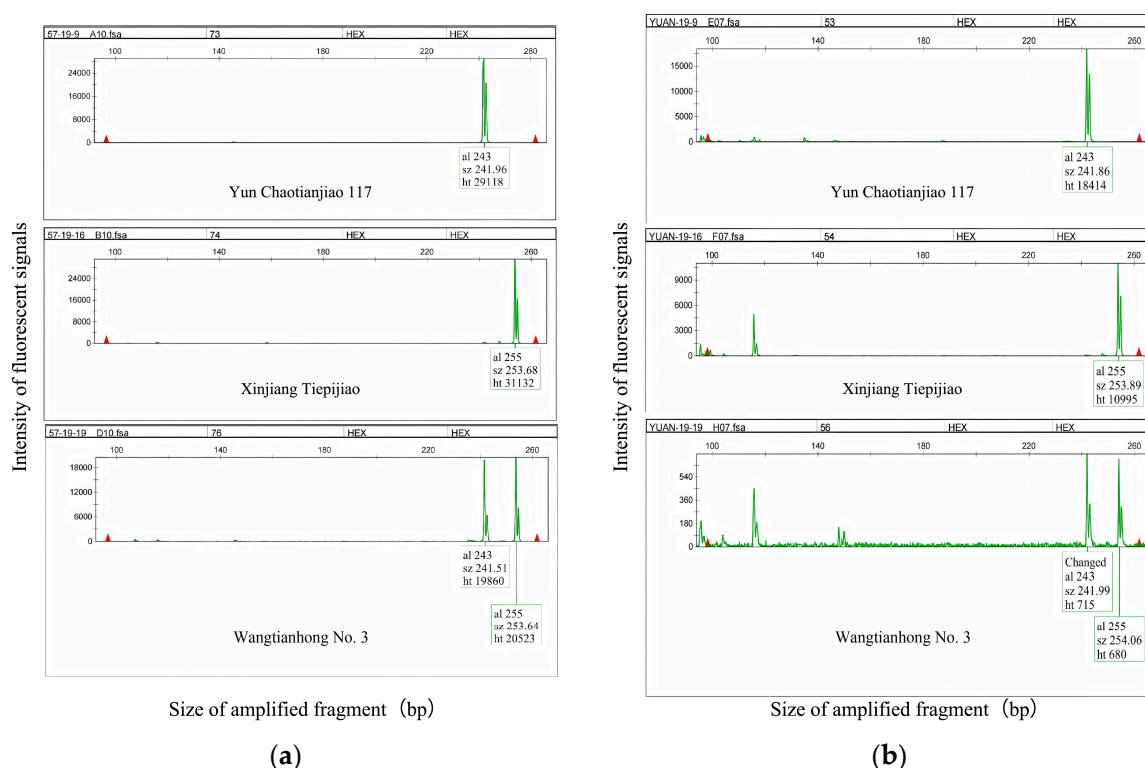


Figure 2. SSR site data reads of ES167 primer at different amplification temperatures in three pepper varieties: (a) 57 °C; (b) the specified temperature in the original NY/T 2475-2013 standard.

Table 3. Information on eight representative varieties.

No.	Variety Name	Characteristics	Region
1	Shuanshuanla	Early maturity, conical fruit, slightly wrinkled skin, for fresh consumption and processing	Yunnan, China
2	Runjianghong No. 16	Early maturity, horn-shaped or elongated fruit, thick flesh, for fresh consumption	Jiangsu, China
3	Yunchaotianjiao 117	Medium-late maturity, conical fruit, smooth skin, for fresh consumption and processing	Yunnan, China
4	Zhouzhoujiao No. 1	Early maturity, long lantern-shaped fruit, wrinkled surface, for fresh consumption	Anhui, China
5	Yunganjiao No. 3	Medium maturity, long finger-shaped fruit, for processing	Yunnan, China
6	Xinjiang Tiepijiao	Late maturity, conical fruit, thick flesh, for processing	Xinjiang, China
7	Zhongjiao 106	Medium-early maturity, ox-horn-shaped fruit, strong surface gloss, for fresh consumption	Beijing, China
8	Wangtianhong No. 3	Early maturity, conical fruit, for fresh consumption and processing	Henan, China

PIC values for the new system ranged from 0.26 to 0.86, averaging 0.49. Compared to the original system, the new system's core primers show higher polymorphism: among the 26 core primer pairs, 14 show moderate polymorphism ($0.25 \leq \text{PIC} < 0.5$), 12 show high polymorphism ($\text{PIC} \geq 0.5$), and none show low polymorphism (Figure 3).

Among them, 24 primer pairs are evenly distributed across the 12 pepper chromosomes, while two are located on different linkage groups. Figure 4 shows the allelic variants detected by the new primer HPMSE128 in four pepper varieties.

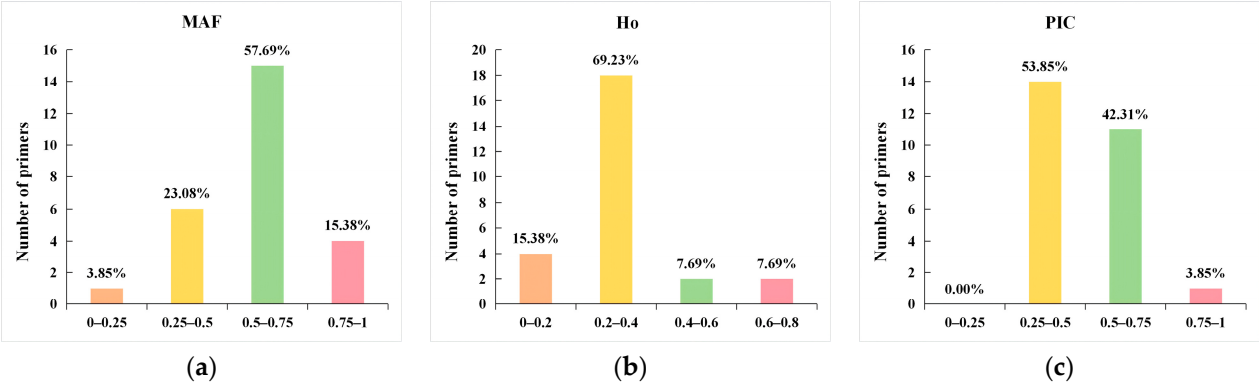


Figure 3. Genetic characteristics of 26 core primer pairs in the new system: (a) main allele frequency; (b) observed heterozygosity; (c) polymorphic information content.

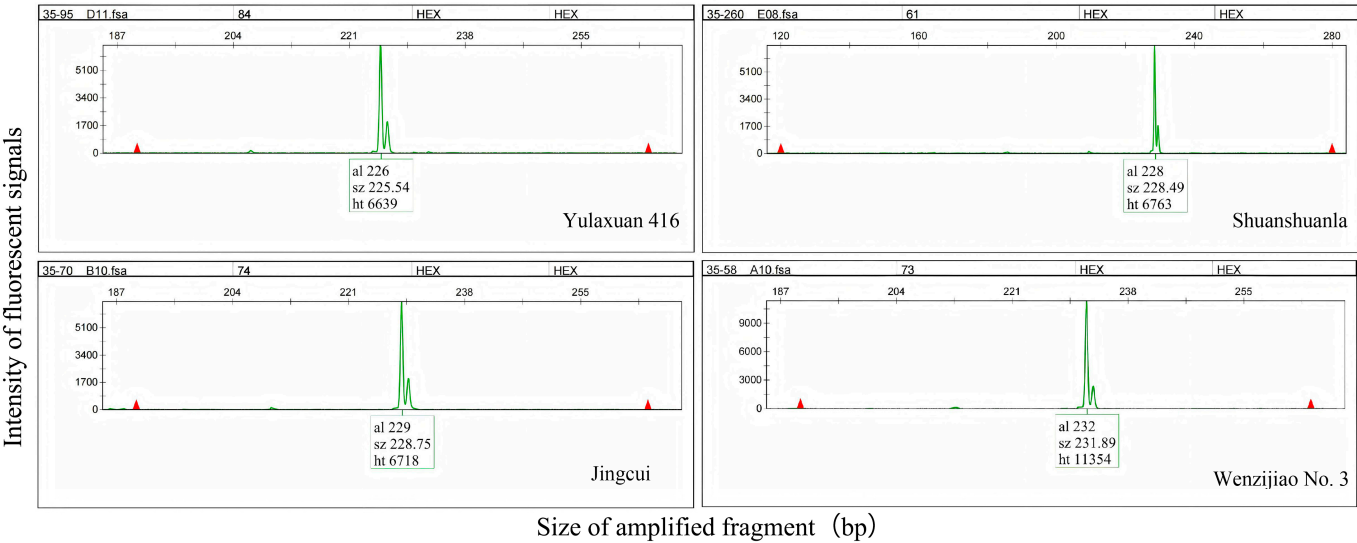


Figure 4. Data reads of HPMSE128 primer at SSR loci in four pepper varieties.

3.2.3. Establishment of the Pepper Variety Identification Technical System and SSR Fingerprint Database

Fingerprints for the 240 pepper varieties were collected using the 26 core primer pairs. Across the 240 pepper varieties, these 26 primer pairs detected 153 alleles and 293 genotypes. The number of alleles per locus ranged from 3 to 15, with an average of 5.9. HPMS1-5 detected the most alleles (15). To eliminate systematic errors between different experimental batches or instrument models, allelic variant sizes were named, and corresponding reference varieties were selected (Table S3) to ensure accurate SSR fingerprint collection. Reference varieties were selected based on ease of propagation and conservation, high intra-variety consistency, and maximizing allele coverage with the fewest varieties. Based on fluorescent dye labels and expected size ranges, the 26 core primer pairs were divided into seven groups for multiplex electrophoresis to improve detection efficiency (Table 4).

Table 4. Grouping of 26 core primer pairs for multiplex fluorescence capillary electrophoresis.

Group	Hexachlorofluorescein	5-Carboxytetramethylrhodamine	6-Carboxyfluorescein	6-Carboxyl-X-Rhodamine
1	ES110 (138–150) HPMSE149 (164–203)	ES417 (73–93)	ES297 (110–122)	CAMS396 (233–247)
2	ES285 (118–121)	HPMS1-214 (82–101)	S82 (191–213) EPMS-923 (253–308)	HPMS1-43 (141–181)

Table 4. Cont.

Group	Hexachlorofluorescein	5-Carboxytetramethylrhodamine	6-Carboxyfluorescein	6-Carboxyl-X-Rhodamine
3	HPMSE015 (132–144) ES167 (237–255)	ES363 (87–107)	SSR82 (157–177)	
4	ES64 (152–168)	S69 (248–267)		ES395 (84–105)
5	ES321 (106–148)	S133 (254–264)	CAMS071 (162–173)	
6	HPMS2-41 (154–164) CAEMS015 (266–271)			ES330 (97–121)
7	HPMSE128 (226–232)		HPMS1-5 (289–320)	ES120 (154–167)

3.3. Evaluation of the New Identification System

3.3.1. Discrimination Efficiency Evaluation

To evaluate the accuracy and efficiency of the new core primer system compared to the original, Nei's genetic distance was analyzed. When the number of differing loci is between 0 and 1, the genetic distance approaches zero, and the system cannot distinguish varieties. Based on these indistinguishable variety combinations, the discrimination efficiency was calculated. Under the original system, genetic distances ranged from 0 to 1 (average 0.39). Among the 28,680 pairwise variety combinations [calculated as the total number of all possible pairs: $C(240, 2) = 240 \times 239/2 = 28,680$] from 240 pepper varieties, three combinations had zero differences and eight had one difference, totaling 11 combinations (0.038%), resulting in a discrimination efficiency of 99.962% (Figure 5a). Under the new system, genetic distances ranged from 0 to 0.98 (average 0.44). Two combinations had zero differences, and five had one difference, totaling seven combinations (0.025%), resulting in a discrimination efficiency of 99.975% (Figure 5b). This indicates that the new system has higher discrimination efficiency. Cluster analysis based on Nei's genetic distance (Figure 5) showed that both systems divided the 240 pepper varieties into six groups. No clear clustering based on province of origin was observed, likely due to frequent germplasm exchange between regions. Notably, Group 1 consistently included *C. frutescens* varieties such as "Jingcui", "Shuanshuanla", and "L515M", which showed greater genetic distance from current commercial varieties, suggesting their potential for use in pepper breeding.

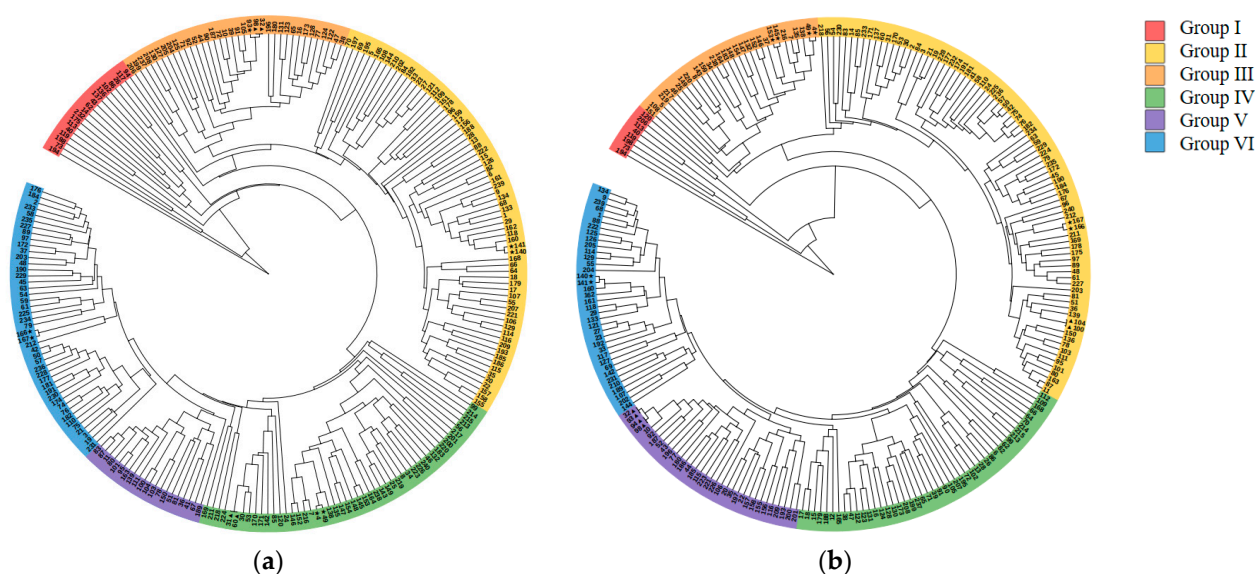


Figure 5. Nei's genetic distance clustering map of 240 pepper varieties. (a) The original system; (b) the new system. Note: ▲ represents 0-locus-difference variety; ★ represents 1-locus-difference variety.

3.3.2. Population Structure Analysis

Population structure analysis of the 240 pepper varieties was performed using SSR fingerprint data to further characterize their population distribution. The $\ln P(K)$ value increased with increasing K values from 1 to 10, with no maximum, making it difficult to determine the optimal number of subpopulations (Figure 6a). Using the method of Evanno et al. [38], the optimal number of subpopulations (K) was determined by identifying a peak in ΔK . The analysis indicated $K = 6$ as the optimal classification (Figure 6b), dividing the 240 pepper varieties into six subpopulations (Figure 6c), consistent with the cluster analysis based on Nei's genetic distance. The population structure plot (Figure 6c) shows significant gene flow between subpopulations, with high admixture. Varieties with a single genetic component were rare; most contained components from more than three subpopulations. This suggests that cross-breeding between subpopulations in pepper breeding may contribute to decreasing genetic differentiation. This finding correlates with the lack of clear provincial clustering in the Nei's genetic distance analysis.

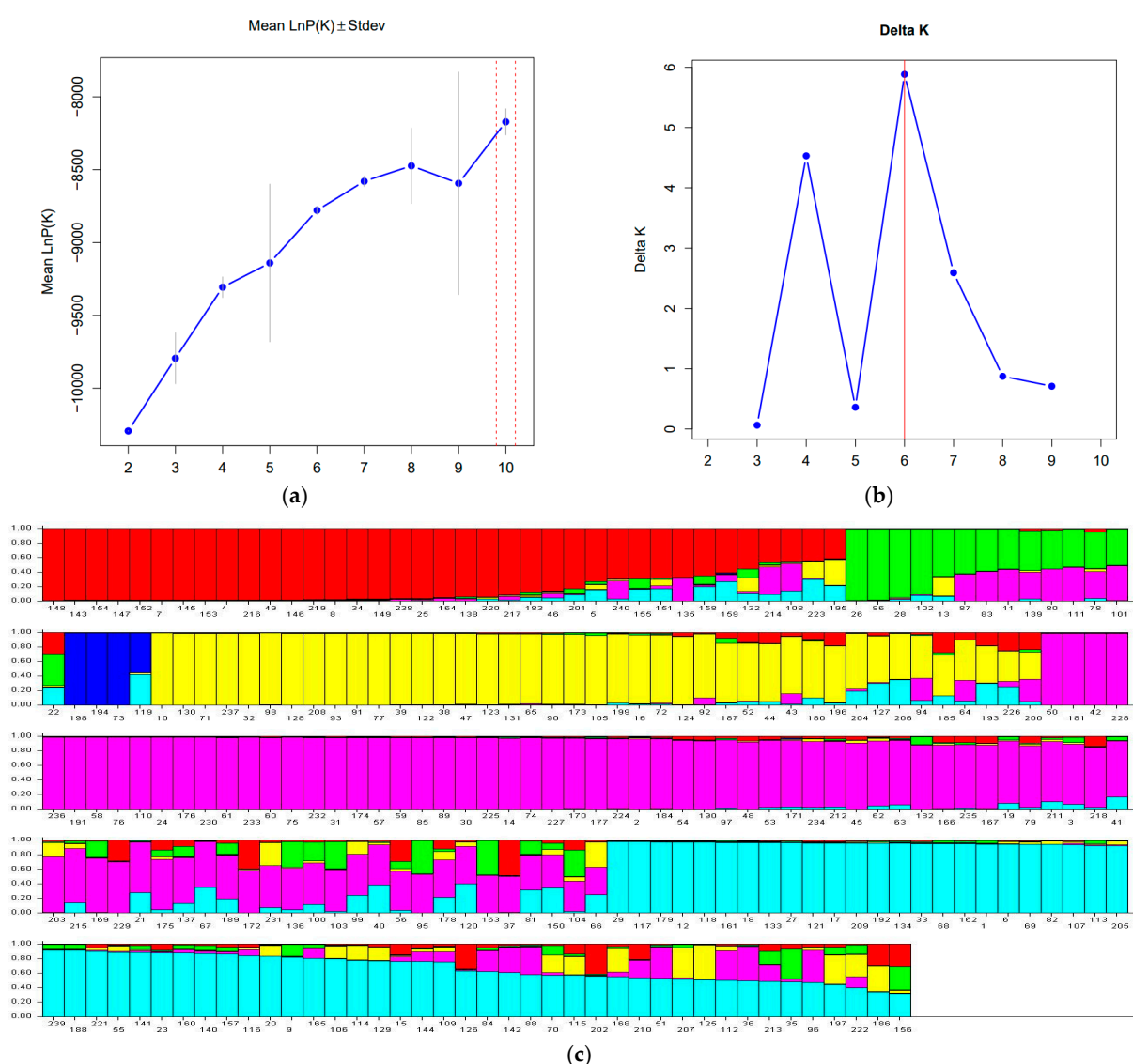


Figure 6. Population structure analysis of 240 pepper varieties based on 26 core primer pairs (new system). (a) The population structure was estimated using the mean value of $\ln P(K)$, with a K value range from 1 to 10. (b) The ΔK curve derived from $\ln P(K)$ was utilized to determine the optimal K value as 6. (c) The 240 pepper varieties were clustered into six subgroups, with each color representing one subgroup.

3.3.3. Application Evaluation of the SSR Fingerprint Database

To explore the feasibility of using the new system to assist in selecting candidate varieties for DUS testing, field trials were conducted with two variety pairs with zero allelic differences, four pairs with one allelic difference, and five candidate varieties provided by applicants. Analysis of 34 phenotypic traits revealed that varieties with closer molecular genetic distances often had the same (Groups 1, 3, 4, 5, and 6) or sister line (Group 2) varieties as candidate varieties provided by applicants, indicating that varieties showing no differences with SSR markers do exhibit some phenotypic similarity (Table 5). Phenotypic similarity among varieties ranged from 0.58 to 1, with an average of 0.68 (Figure 7). The phenotypic similarity between “Beike 20” and the zero-difference variety “H12119A” was 0.82, while similarity between “Beike 20” and its applicant-provided candidate variety “Sanyingbahao” was 0.74. Similarly, similarity between “Surun 600” and the one-difference variety “Surun 858” was 0.78, compared to 0.76 with its candidate variety “Sugar No. 1”. For “Tianlong 29,” similarity with the one-difference variety “Tianyi Liujiao” was 0.71, compared to 0.66 with its candidate variety “Jinta”. Based on phenotypic similarity, varieties selected via molecular markers appear more suitable as candidate varieties for DUS testing, suggesting that the new system can aid in candidate variety selection for DUS.

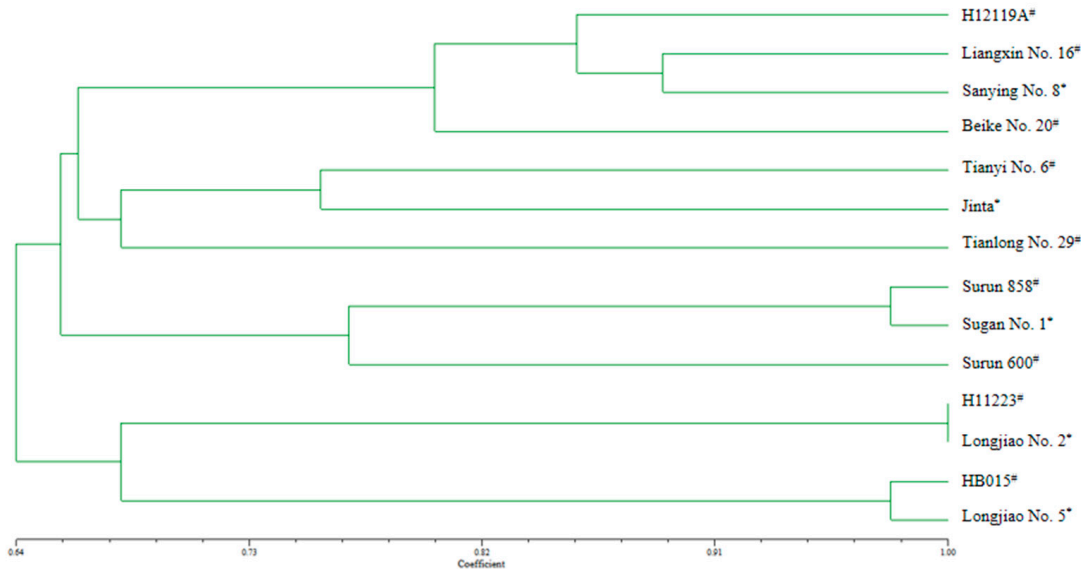


Figure 7. Genetic similarity analysis of 0/1 difference-locus variety pairs and applicant-supplied approximate varieties. Note: # represents 0 or 1 differential site varieties; * represents similar varieties provided by breeders.

Table 5. Field planting experiment grouping: variety pairs with 0/1 difference loci and applicant-supplied approximate varieties.

Group	Variety No.	Variety Name	Variety Relationship to Candidate Variety
1	32	H12119A	0 variant loci
	98	Beike 20	0 variant loci
		Sanying No. 8	Applicant for similar variety (Beike 20)
2	31	H11223	0 variant loci
		Longjiao No. 2	Applicant for similar variety (H11223)
	60	HB015	0 variant loci
3		Longjiao No. 5	Applicant for similar variety (HB015)
	93	Liangxin No. 16	1. Varieties with differential loci
	32	H12119A	1. Varieties with differential loci
		Sanying No. 8	Applicant for similar variety (Liangxin No. 16)

Table 5. Cont.

Group	Variety No.	Variety Name	Variety Relationship to Candidate Variety
4	93	Liangxin No. 16	1. Varieties with differential loci
	98	Beike 20	1. Varieties with differential loci
		Sanying No. 8	Applicant for similar variety (Liangxin No. 16, Beike 20)
5	140	Su Run 858	1. Varieties with differential loci
	141	Su Run 600	1. Varieties with differential loci
		Sugan No. 1	Applicant for similar variety (Su Run 858, Su Run 600)
6	4	Tianyi No. 6	1. Varieties with differential loci
	49	Tianlong 29	1. Varieties with differential loci
		Jinta	Applicant for similar variety (Tianyi No. 6, Tianlong No. 29)

4. Discussion

In variety molecular identification systems, factors such as the polymorphism information content (PIC) of core primers, their chromosomal distribution, amplification stability, and locus heterozygosity are highly correlated with discriminatory ability. The range of annealing temperatures and the use of multiplex electrophoresis are related to the efficiency of the core primer system. Reference varieties are crucial for minimizing platform-related errors [39–41]. In this study, the 26 SSR core primer pairs included 14 with moderate polymorphism and 12 with high polymorphism, with no low-polymorphism primers. They were evenly distributed across the 12 pepper chromosomes, amplified stably, produced clear peaks, and could distinguish 229 out of 240 pepper varieties, achieving a discrimination efficiency of 99.975%. This meets the requirement of the “General Principles for Plant Variety Identification Using DNA Molecular Marker Methods” that core primers should distinguish over 95% of varieties (NY/T 2594-2016), demonstrating strong discriminatory power. The uniform annealing temperature of 57 °C and the formation of seven multiplex fluorescent capillary electrophoresis groups ensure the system’s high efficiency. Recent studies by Yi et al. [42] confirmed that fluorescent capillary electrophoresis platforms significantly enhance the throughput and reproducibility of SSR-based variety identification in vegetable crops, aligning with our findings. Additionally, Anani et al. [43] demonstrated that optimized core primer sets with high PIC values (>0.5) could achieve discrimination efficiencies exceeding 99.9% in pepper, which is consistent with our results. The selection of 42 reference varieties and the high precision of fluorescent capillary electrophoresis help reduce systematic errors, enhancing the accuracy of the core primer system. Overall, the performance metrics of this system surpass those of the original, making it more suitable for pepper variety identification.

Notably, three SSR markers, HPMS1-5, HPMS1-214, and HPMS1-106, which originated from the original standard system (NY/T 2475-2013), could not be mapped to the current chromosome-level reference genome of pepper through sequencing and BLAST alignment. This is likely the combined result of structural discordance between genetic and physical maps, regional gaps in the reference genome assembly, and the intrinsic sequence characteristics of these markers. Although HPMS1-5 and HPMS1-214 were previously assigned to LG6 and LG1, respectively, in the SNU2 genetic map [37], this map, derived from an interspecific cross (*C. annuum* cv TF68 × *C. chinense* cv Habanero), exhibits collinearity breaks, and the presence of 15 linkage groups instead of the 12 chromosome pairs indicates a discrepancy between genetic and physical positions [37]. Moreover, over 80% of the pepper genome consists of repetitive sequences, and LTR retrotransposon-rich regions are particularly prone to assembly gaps [31,44]; the flanking sequences of the HPMS markers are themselves homologous to transposable elements [37], suggesting that their locations likely fall into assembly blind spots. HPMS1-106 was not even localized in the original genetic map [37], further supporting its residence in highly heterochromatic regions. In ad-

dition, interspecific structural variations [23,44] and the high PIC values leading to flanking sequence polymorphisms [1] may have reduced the sensitivity of BLAST alignment [44].

Although none of the three markers HPMS1-5, HPMS1-214, and HPMS1-106 could be physically assigned to the current pepper reference genome, polymorphism evaluation revealed that HPMS1-5 and HPMS1-214 possessed high PIC values of 0.86 and 0.58, respectively, and were therefore retained in the new system; HPMS1-106, however, with a PIC value of only 0.46 and no localization in the original genetic map, was excluded. The new optimized system achieved marker distribution across all 12 chromosomes, and the failure to localize HPMS1-5 and HPMS1-214 did not compromise the overall balance of chromosomal coverage. The final marker set exhibited a cultivar discrimination efficiency of 99.975% and an average PIC value of 0.49, indicating that the selected markers captured sufficient genome-wide genetic variation to enable reliable cultivar discrimination. Therefore, in practical applications, marker polymorphism information content and amplification reproducibility are more critical than complete chromosomal physical coverage. Nevertheless, future studies should fully exploit recently released chromosome-level genome assemblies and pepper pan-genome resources to develop chromosome-specific SSRs or integrate complementary SNP and InDel markers, thereby further improving genome coverage and enhancing the robustness of the molecular identification system.

Constructing crop variety DNA fingerprint databases using molecular markers for authenticity identification has become a crucial technical tool for variety management, identification, and rights protection globally. In recent years, the Chinese Ministry of Agriculture and Rural Affairs has actively promoted the development and application of DNA fingerprinting in the seed industry to enhance scientific detection capabilities. A fingerprint detection system based on internationally accepted SSR and SNP markers has been established, facilitating the construction of DNA fingerprint databases for 34 crop species, including corn [41], rice [45], and wheat [46], which are used in national seed market inspections. Harishkumar et al. [47] used nine SSR markers to fingerprint 37 pepper varieties. Lei et al. [48] used 49 SSR markers to fingerprint 60 key pepper parent lines. This study uses a larger number of varieties with clearer origins than previous fingerprint database research. Two common methods for constructing DNA fingerprint databases using SSR markers are based on PAGE and capillary electrophoresis. Hao et al. [49] compared these methods in wheat, finding capillary electrophoresis to be 7–8 times more efficient. This study employed fluorescent capillary electrophoresis to obtain amplicon fragment sizes and constructed a DNA fingerprint database comprising 240 pepper varieties—a more intuitive, convenient, and efficient approach. Zhang et al. [50] recently emphasized that high-throughput fingerprint databases based on SSR markers are essential for variety rights protection and seed market regulation, supporting our approach. Currently, almost all national SSR-based variety identification standards use amplicon fragment size as the database specification. The DNA fingerprint database constructed in this study meets these standard requirements and is highly applicable.

DUS testing (Distinctness, Uniformity, and Stability) is essential for determining whether a candidate plant variety qualifies for protection [51]. Correct selection of candidate varieties is critical for assessing distinctness. Selecting candidate varieties based solely on phenotypic traits is environmentally sensitive and challenging without a comprehensive phenotypic database, potentially leading to incorrect selections [52–54]. Yin et al. [7] found a moderate correlation between molecular markers and phenotypic traits, suggesting that molecular markers can aid candidate variety selection. Combining field phenotypic assessment with molecular marker analysis can make variety identification more accurate and efficient, a practice also used in germplasm identification and genetic diversity analysis [8]. In this study, varieties with molecular genetic distances approach-

ing zero exhibited high phenotypic similarity. Furthermore, for varieties with molecular distances near zero, the candidate varieties provided by applicants were often the same or sister lines. This indicates that the results of genetic diversity analysis using this core primer system correlate with phenotypic classification, demonstrating its applicability and effectiveness in assisting candidate variety selection for DUS. Kwon et al. recently reported that SSR markers significantly improve the accuracy of candidate variety selection in DUS testing for *Capsicum* species, reducing field trial costs and time, which directly supports our conclusions [53]. Similarly, Bukhari et al. [54] found that molecular marker-based genetic distance correlates well with phenotypic distinctness in Chinese pepper varieties, reinforcing the practical utility of our system.

Genetic diversity, resulting from gene mutation, recombination, and chromosomal aberrations, determines biodiversity. Molecular markers, unaffected by environment, are essential for genetic diversity analysis. Studying the genetic diversity of pepper germplasm helps understand genetic stability, evolutionary capacity, and environmental adaptability, informing strategies for germplasm conservation and breeding [55]. In this study, cluster analysis based on Nei's genetic distance divided the 240 pepper varieties into six groups without clear provincial clustering. This suggests that with increasing breeding exchanges, the types of peppers used across regions are becoming more diverse, but the overall genetic differences are narrowing. The genetic background of peppers across regions is becoming increasingly narrow. Population structure analysis of the 240 pepper varieties also revealed six subpopulations with significant gene flow. While this differs somewhat from Gu et al. [19], who identified two main genetic groups with admixture in Chinese peppers, it is consistent with Nicolai et al. [56], who found six major genetic groups in *Capsicum* collections from 89 countries. This suggests that the pepper varieties used in this study are representative, and the identification system developed based on them is broadly applicable. Notably, Group 1 in the cluster analysis included *C. frutescens* varieties like "Jingcui", "Shuanshuanla", and "L515M", which are genetically distant from current commercial varieties, representing valuable germplasm resources for future pepper breeding. Hill et al. [57] reported that extensive cross-breeding and gene flow among pepper subpopulations have led to reduced genetic differentiation in many Asian countries, which aligns with our observation of high admixture among Chinese pepper varieties. Furthermore, Khan et al. [58] demonstrated that whole-genome-based SSR marker optimization can capture fine-scale genetic diversity patterns that were previously undetected, validating our approach of using an improved core primer set for diversity analysis.

5. Conclusions

A new pepper variety identification system was developed, comprising 26 core primer pairs: 12 newly screened and 14 retained from the original standard. Based on a fluorescent capillary electrophoresis platform, a DNA fingerprint database containing 240 pepper varieties was constructed. Combined with phenotypic data, the new core primer system distinguished 229 out of 240 pepper varieties, achieving a discrimination efficiency of 99.975%, meeting the requirements of the "General Principles for Plant Variety Identification Using DNA Molecular Marker Methods". This system is applicable for authenticity and purity identification of pepper varieties, parentage testing of hybrids, assisting in candidate variety selection for DUS testing, and supporting the protection of new variety rights.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12070848/s1>, Table S1: Information on 240 pepper varieties; Table S2: The complete list of the 150 primer pairs included in the screening; Table S3: Allelic variation and reference varieties for 26 core primer pairs.

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