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Fluorescent SSR-Based DNA Fingerprinting and Molecular Identity Card Development for 69 Mandarin Accessions

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Abstract

To establish standardized DNA fingerprinting and molecular identification systems for citrus, we analyzed 69 mandarin accessions via fluorescent SSR capillary electrophoresis to construct DNA molecular fingerprints and unique molecular identity cards. Eighteen highly polymorphic SSR primer pairs were screened, yielding 239 genotype calls and 147 alleles. The number of amplified alleles per primer pair ranged from 4 to 18, with polymorphic information content (PIC) values varying from 0.411 to 0.650. Ten core primer pairs were further selected, achieving a discrimination rate of 65.2% (45 out of 69 accessions distinguished). Utilizing these fluorescent SSR markers, we established DNA molecular fingerprints and unique molecular identity cards for all 69 accessions. Among them, 45 accessions possessed unique fingerprints, whereas the remaining 24 indistinguishable accessions were clustered into six groups. Each cluster contained both wild (4 accessions total) and cultivated (20 accessions total) resources with high genetic similarity, which merits further investigation. This study lays a theoretical basis for the authentication, conservation, and genetic relationship analysis of mandarin germplasm resources, and provides a practical tool for standardizing mandarin variety identification.

Keywords: citrus; capillary electrophoresis; DNA fingerprint; molecular identity card

1. Introduction

Mandarin citrus (*Citrus reticulata* Blanco), commonly known by the Chinese names "huangguo" and "guanggan", is distinguished by its loosely adherent peel and encompasses a broad spectrum of tangerine and mandarin cultivars [1]. As the most extensively cultivated citrus group in China, mandarins exhibit substantial genetic diversity [2], with their taxonomic framework anchored in the seminal work of Barrett and Rhodes, who delineated just three true *Citrus* species: *C. medica* (citron), *C. grandis* (pummelo), and *C. reticulata* (mandarin) [3]. China serves as both a primary center of origin and a genetic diversity hotspot for mandarins [4,5], harboring a rich assemblage of cultivars—including Ponkan, Satsuma mandarin, Shatangju, and Nanfeng tangerine—that encompass both monoembryonic and polyembryonic genotypes [6].

Traditional identification of citrus cultivars relies heavily on morphological traits, a practice that proves inherently challenging for accessions with highly similar leaf morphology, owing to strong subjectivity and susceptibility to environmental interference. To circumvent these limitations, modern molecular biological techniques have emerged as powerful tools, enabling convenient,

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accurate, and reliable cultivar discrimination at the DNA level. Among these techniques, simple sequence repeat (SSR) markers are particularly valued for their high polymorphism, excellent reproducibility, and codominant inheritance patterns [7–9]. Nevertheless, conventional SSR genotyping based on polyacrylamide gel electrophoresis (PAGE) is plagued by operational cumbersomeness, the use of toxic reagents, low detection efficiency, and an inability to precisely quantify fragment sizes—drawbacks that constrain its applicability in large-scale germplasm analysis.

Fluorescent capillary electrophoresis (FCE) technology has been developed to overcome the limitations of traditional PAGE-based SSR detection, enabling stable, high-throughput, and precise determination of amplified fragment sizes. While FCE-coupled SSR analysis has been successfully deployed for DNA fingerprinting, molecular identity (ID) development, and cultivar authentication in major crops such as maize and rice [10–13], its application in citrus remains relatively limited. Advances in molecular marker technologies have further solidified the status of SSR markers as pivotal tools for citrus cultivar authentication, with numerous studies demonstrating their utility across diverse citrus research contexts [14,15]. For instance, Rohini et al. [16] employed 17 SSR loci to detect minimal genetic differentiation within Indian citrus germplasm; Yan et al. [17] validated genetic stability between ‘Guanxi’ pomelo and its bud sports using 15 SSR markers; and Lidija et al. [18] confirmed the efficacy of SSRs for citrus genotype characterization. Beyond genetic diversity assessments, SSR markers have been applied to practical citrus research: Fan et al. [19] established protocols for citrus seedling purity testing; Zeng et al. [20] developed cultivar-specific fingerprints for Nanfeng tangerine; Lei et al. [21] identified 12 polymorphic PAGE-based SSR primers for constructing citrus fingerprint databases; Li et al. [22] built a fingerprint library covering 500 citrus accessions using FCE; Biswas et al. [23] developed genome-derived SSR markers for citrus authentication; and Chen et al. [24] generated DNA fingerprints for the hybrid cultivar ‘Zhonggan No.5’ (a cross of ‘Egan No.30’ × ‘Shatangju’).

Despite mandarins ranking as the second most consumed fresh citrus fruit in China, standardized SSR-based molecular identification systems tailored specifically for this group remain inadequately developed. Against this backdrop, the present study aims to establish DNA fingerprints and molecular ID cards for 69 mandarin accessions using FCE-based SSR markers. This work provides a foundational reference framework for standardized varietal authentication, germplasm conservation, and genetic relationship analysis of mandarins and their related citrus taxa, thereby supporting the sustainable development of the global citrus industry.

2. Materials and Methods

2.1. Materials

Sixty-nine mandarin accessions (detailed in Table 1) were collected from the Citrus Germplasm Resource Preservation Nursery of the Guangxi Academy of Specialty Crops. All materials were propagated and maintained via grafting to ensure genetic consistency. Fresh tissue samples were collected and immediately stored at −80 °C to preserve DNA integrity for subsequent analysis.

2.2. DNA extraction

Genomic DNA was extracted from the stored tissues following the method described by Lin and Walker [25], with minor modifications. DNA quality was evaluated using 1.2% agarose gel electrophoresis (stained with ethidium bromide) to assess for degradation and contamination. The purity (A_{260}/A_{280} ratio) and concentration of extracted DNA were determined using a nucleic acid-protein quantifier. Only DNA samples with an A_{260}/A_{280} ratio of 1.8–2.0 (indicating high purity) were used for subsequent SSR amplification.

2.3. Primer design and synthesis

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SSR loci were identified from the clementine (*Citrus reticulata*) genome sequence. The minimum number of repeats for SSR loci was set as follows: 6 repeats for mononucleotide motifs, and 5 repeats each for di-, tri-, tetra-, penta-, and hexanucleotide motifs. Specific primers targeting these SSR loci were designed using Primer 5.0 software, adhering to the following criteria: primer length of 20–24 bp, GC content of 40–60%, annealing temperature (Tm) of 57–62 °C, and expected amplicon size of 100–350 bp. All primers were synthesized by a commercial biotechnology company, with the 5′ end of each forward primer labeled with a fluorescent dye (FAM, HEX, or ROX) to facilitate subsequent capillary electrophoresis detection.

2.4. Primer Screening

A three-step primer screening strategy was adopted to select high-quality polymorphic primers:

1. **Preliminary screening**: Primers were initially tested via 2% agarose gel electrophoresis using genomic DNA from 8 randomly selected mandarin accessions. Each PCR reaction was performed in duplicate to verify reproducibility. Primers that yielded clear, non-smearing, and reproducible bands were selected for secondary screening.
2. **Secondary screening**: Candidate primers from the preliminary step were further evaluated using 6% denaturing polyacrylamide gel electrophoresis (PAGE) to assess polymorphism. Each reaction was repeated twice, and primers exhibiting distinct polymorphic bands among the test accessions were retained.
3. **Final validation**: Primers with satisfactory performance in secondary screening were subjected to final validation using SSR fluorescent capillary electrophoresis (FCE). This step confirmed their polymorphism, amplicon stability, and suitability for large-scale fingerprinting analysis of the 69 mandarin accessions.

Table 1. List of 69 experimental accessions.

Code	Accession name	Scientific Name	Code	Accession name	Scientific Name
1	Guposhanyeju	*Sinocitrus chuana*	36	Yingxinju	*C. reticulata*
2	Xinganyeju	*C. reticulata*	37	Pixeju	*C. reticulata*
3	Daoxianyeju	*C. daoxianensis*	38	Bendizao	*C. reticulata*
4	Mangshanyeju	*C. reticulata*	39	Penggan No79-2	*C. reticulata*
5	Niuduyeju	*C. reticulata*	40	Dong No13penggan	*C. reticulata*
6	Hezhouyeju	*C. reticulata*	41	Shi18penggan	*C. reticulata*
7	Yinduyeju	*C. indica*	42	Taitianpenggan	*C. reticulata*
8	Lihuaju	*C. tachibana*	43	Xinshengxipenggan No3	*C. reticulata*
9	Cengxisuanju	*C. reticulata*	44	Wuhepenggan	*C. reticulata*
10	Guangxihongpisuanju	*C. reticulata*	45	Dafen No4	*C. reticulata*
11	Guposhanchougan No2	*C. reticulata*	46	Rinan No1	*C. reticulata*
12	Guposhanchougan No5	*C. reticulata*	47	Dapu No5	C. reticulata
13	Guposhanchougan No6	*C. reticulata*	48	Miyamoto	C. reticulata
14	Yuanyemangsha	*C. mangshanesis*	49	Miyagawa	C. reticulata

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15	nyegan Jianyemangshan yegan	*C. mangshanesis*	50	Hashikawa	C. reticulata
16	Shagan	*C. nobilis* Lour	51	Xingjin	C. reticulata
17	Biangan	*C. reticulata*	52	Yoshida	C. reticulata
18	Banyeshenggan	*C. reticulata*	53	Ichibun	C. reticulata
19	Huangpisuanju	*C. reticulata*	54	Yamasitabeni	C. reticulata
20	Hongpisuanju	*C. reticulata*	55	Katsuyamano	C. reticulata
21	Shatangju	*C. reticulata*	56	Ueno	C. reticulata
22	Zaoshushatangju	*C. reticulata*	57	Dajin No4	C. reticulata
23	Yamada	*C. reticulata*	58	Zaoxiang	C. reticulata
24	Bayueju	*C. reticulata*	59	Sakikubo	C. reticulata
25	Denglongju	*C. reticulata*	60	Jinzhixiang	C. reticulata
26	Jinkuimiju	*C. reticulata*	61	Youliang	C. reticulata
27	Nanfengmiju1	*C. reticulata*	62	Chunjian	C. reticulata
28	Nanfengmiju2	*C. reticulata*	63	Nanxiang	C. reticulata
29	Tezaoshumiju	*C. reticulata*	64	Murcott	C. reticulata
30	Liuchengmiju	*C. reticulata*	65	Gonggan	C. reticulata
31	Guijuyihao	*C. reticulata*	66	Wogan	C. reticulata
32	Clementine	*C. reticulata*	67	Huangmeiren	C. reticulata
33	Mingliutianju	*C. reticulata*	68	Aiyuan No38	C. reticulata
34	Chuntianju	*C. reticulata*	69	Mingrijian	C. reticulata
35	Guangxiju	*C. reticulata*			

2.5. Fingerprint Construction

Validated genotypes obtained from SSR primer amplification were used for DNA fingerprint construction. For each primer pair, the molecular weights of amplified alleles were sorted in descending order and assigned unique Arabic numerals (e.g., 1, 2, 3...) as genotype codes. An Excel-based DNA fingerprint map was generated, where the x-axis represented the molecular weights of amplified alleles for each primer pair, and the y-axis corresponded to the 69 mandarin accessions. This map provided an intuitive visualization of the DNA fingerprint profiles for all tested samples, enabling direct differentiation of accessions based on banding pattern differences.

2.6. Molecular Identity Card Construction

To establish standardized molecular identity (ID) cards, the amplified allele sizes of each primer pair were converted into unified numeric or alphabetic codes following a predefined rule:

1. For each primer pair, the fragment sizes of amplified alleles were first sorted in ascending order;
2. Unique band patterns (genotypes) among the 69 accessions were encoded sequentially using Arabic numerals 1–9;
3. When the number of unique band patterns exceeded 9, uppercase English letters (A, B, C, ...) were used to represent the 10th, 11th, 12th, and subsequent patterns;
4. Null alleles (no amplification products) were denoted as "0".

Finally, the codes corresponding to each selected core primer pair were concatenated in a fixed order to form a unique molecular ID card for each mandarin accession.

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3. Results and Analysis

3.1. Primer Design and Screening

Specific SSR primers were successfully designed based on the *Citrus reticulata* clementine genome, resulting in the synthesis of 96 novel primer pairs. To expand the primer pool, these 96 newly designed pairs were combined with 46 previously reported SSR primer pairs for subsequent screening. PCR amplification was first performed using genomic DNA from 6 randomly selected citrus accessions. Through a three-step screening process—2% agarose gel electrophoresis (for preliminary band quality verification), 6% denaturing polyacrylamide gel electrophoresis (for polymorphism preliminary evaluation), and fluorescent capillary electrophoresis (for final validation)—70 primer pairs with strong amplification stability and distinct polymorphism were identified for subsequent experiments.

3.2. Genetic Diversity Analysis of 69 Mandarin Accessions

3.2.1. Polymorphism Evaluation of SSR Primers

Fluorescent capillary electrophoresis analysis was conducted on the 69 mandarin accessions using the 70 screened primer pairs. Among these, 18 primer pairs exhibited significant polymorphism and stable amplification efficiency, and were thus selected for detailed genetic diversity analysis. The amplified fragment sizes ranged from 122 to 369 bp, with a total of 147 alleles detected across all 18 loci. The average number of alleles per primer pair (Na) was 8.16, with allele counts per locus varying from 4 to 18. Primer S90 produced the highest number of alleles, while primers S76 and S85 yielded the fewest. A total of 239 genotypes (amplified bands) were identified from the 18 primer pairs, with 6–30 genotypes per locus. Notably, the number of genotypes exceeded the number of alleles for all 18 primer pairs, indicating high heterozygosity and polymorphism of these loci.

The effective number of alleles (Ne) ranged from 1.883 to 6.089, reflecting substantial variation in allele frequency and potential functional importance of these loci. Shannon's information index (I), a key indicator of genetic diversity, averaged 1.410 across the 18 primer pairs, with 9 pairs showing values above this average. The average observed heterozygosity (Ho) was 0.532, confirming high genetic diversity within the 69 mandarin accessions.

Polymorphic information content (PIC) values were used to classify primer polymorphism: primers with PIC > 0.5 were defined as highly polymorphic, and those with 0.4 ≤ PIC ≤ 0.5 as moderately polymorphic. The average PIC value of the 18 primer pairs was 0.621, with 9 pairs exceeding this average. All 18 primers exhibited PIC values ≥ 0.411, among which 12 were highly polymorphic and 6 were moderately polymorphic, indicating that these primers carry rich polymorphism information and are suitable for mandarin genetic diversity analysis and fingerprint construction (Table 2).

Table 2. Amplification information for 18 SSR primers.

Primer	Amplified bands	Na	Ne	I	Ho	He	PIC	Size range(bp)
S17	19	9	6.089	1.884	0.833	0.836	0.814	166～200
S81	15	10	5.678	1.916	0.507	0.824	0.802	167～192
S90	30	18	4.784	2.109	0.529	0.791	0.776	148～202
S21	19	11	4.535	1.780	0.638	0.78	0.751	244～269
S70	18	14	4.411	1.854	0.783	0.773	0.747	218～248
S01	13	6	3.492	1.462	0.493	0.714	0.677	282～307
S11	10	6	2.346	1.16	0.492	0.574	0.539	172～191
S13	8	6	2.537	1.111	0.381	0.606	0.527	230～251
S28	9	6	2.836	1.239	0.319	0.647	0.582	308～322
S18	15	9	3.749	1.517	0.681	0.733	0.689	230～249
S23	12	8	2.260	1.248	0.435	0.558	0.535	139～189

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S71	13	6	3.614	1.438	0.681	0.723	0.681	211～237
S73	9	7	2.130	1.141	0.529	0.53	0.503	344～369
S76	6	4	2.073	0.81	0.696	0.518	0.411	168~186
S74	8	6	2.383	1.151	0.338	0.58	0.538	182～203
S82	17	9	3.084	1.437	0.368	0.676	0.622	122～158
S84	10	8	2.557	1.225	0.471	0.609	0.561	265～292
S85	8	4	1.883	0.889	0.406	0.469	0.432	125~137

3.2.2. SSR Characteristic Fingerprint Information of 69 Mandarin Varieties

Among the 69 mandarin accessions, 29 possessed unique alleles that were not detected in other accessions. These unique alleles could serve as diagnostic markers for distinguishing these accessions from others. The number of specific unique alleles varied among these 29 accessions, providing a basis for their rapid and accurate identification (Table 3).

3.2.3. Cluster Analysis

Based on polymorphism level, stability, and amplification efficiency, 10 primer pairs (S01, S11, S13, S17, S18, S21, S73, S76, S85, and S90) were selected as core primers for cluster analysis. A phylogenetic tree was constructed based on genetic distance using the unweighted pair-group method with arithmetic means (UPGMA), which clustered the 69 mandarin accessions into three distinct groups: Group I contained a single accession, 'Yinduyeju'; Group II included 5 accessions, such as 'Guposhan wild tangerine' and 'Mangshan wild tangerine'; Group III was the largest group, comprising 63 accessions, which were further subdivided into four subgroups. The clustering results were generally consistent with traditional citrus taxonomic classifications, reflecting the genetic relationships among different mandarin germplasms. Additionally, genetic differences were observed between certain wild accessions and cultivated hybrids, which may be attributed to the absence of distantly related germplasms (e.g., kumquat (*Fortunella* spp.) and trifoliate orange (*Poncirus trifoliata*)) in this study.

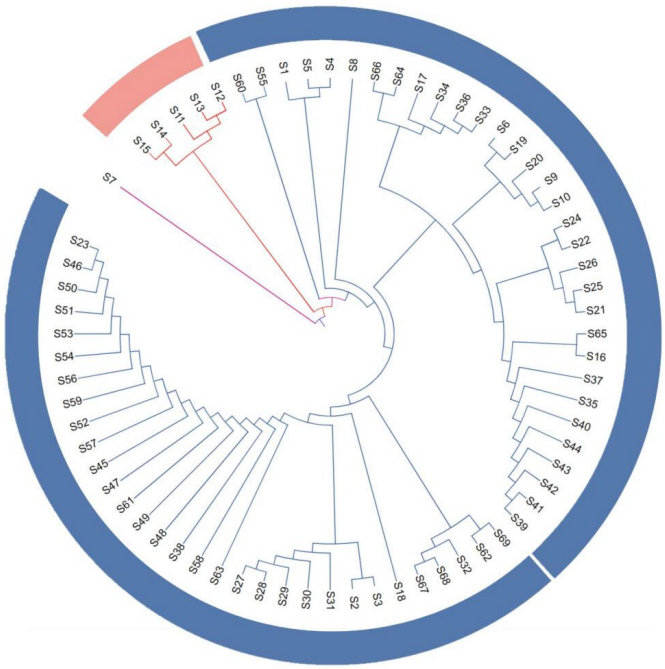

Figure 1. Dendrogram of 69 *C.reticulata* varieties based on SSR markers using UPGMA.

3.3. Construction of DNA Fingerprint and Molecular Identity Card

3.3.1. DNA Fingerprint Construction

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DNA fingerprints for the 69 mandarin accessions were constructed based on the validated genotypes and allele molecular weights determined by fluorescent capillary electrophoresis. Figure 2 presents the comprehensive DNA fingerprint map, where the vertical axis corresponds to the 69 mandarin accessions (with amplified alleles at each SSR locus), and the horizontal axis represents the molecular weights of amplified fragments across all tested loci. This map intuitively displays the distinct banding patterns of each accession, laying a foundation for rapid varietal discrimination.

Table 3. SSR primers containing specific alleles.

Primer	Number	Accession name	Idiotype	Primer	Number	Accession name	Idiotype	Primer	Number	Accession name	Idiotype
S01	2	Yinduyeju	307/307			Guposhanyeju	230/232			Guposhanchougan No5	170/170
		Lihuaju	282/283			Hezhouyeju	226/240	S84	3	Yuanyemangshanyegan	279/279
S11	2	Yinduyeju	179/179	S70	7	Yinduyeju	228/228			Jianyemangshanyegan	277/285
S13	2	Biangan	188/191			Lihuaju	224/224			Guposhanyeju	165/171
		Lihuaju	230/245			Huangpisuanju	234/248			Mangshanyeju	161/165
		Yinduyeju	251/251			Guijuyihao	230/244			Nieduyeju	161/171
S17	5	Biangan	170/170			Aiyuan No38	220/226			Hezhouyeju	152/164
		Banyeshenggan	166/170			Mangshanyeju	219/234			Lihuaju	166/202
		Zaoxiang	180/184			Yinduyeju	211/237			Guposhanchougan No2	148/150
		Wogan	180/180	S71	4	Clementine	237/237			Guposhanchougan No5	150/173
		Aiyuan No38	170/198			Gonggan	219/219			Guposhanchougan No6	148/173
S18	6	Lihuaju	247/249	S74	2	Yinduyeju	191/191			Shagan	158/172
		Guposhanchougan No5	233/233			Huangpisuanju	182/203	S90	19	Biangan	172/177
		Yuanyemangshanyegan	246/246			Guposhanyeju	169/174			Huangpisuanju	161/164
		Jianyemangshanyegan	243/246	S81	5	Mangshanyeju	173/176			Kelimandingju	158/161
S21	5	Katsuyamano	231/239			Hezhouyeju	169/178			Guangxiju	161/161
		Youliang	237/243			Yinduyeju	170/170			Pixeju	161/173
		Yinduyeju	244/244			Bendizao	178/187			Bendizao	159/166
		Lihuaju	251/267			Nieduyeju	140/147			Katsuyamano	157/161
		Biangan	255/259			Yinduyeju	147/147			Jinzhixiang	157/166
		Banyeshenggan	267/269			Lihuaju	122/150			Gonggan	158/166
		Bendizao	259/259			Yuanyemangshanyegan	140/153			Aiyuan No38	173/173
S23	6	Guposhanyeju	163/163			Jianyemangshanyegan	153/153	S76	2	Yinduyeju	168/168
		Nieduyeju	163/169			Huangpisuanju	137/137			Mangshanyeju	180/186
		Hezhouyeju	169/189	S82	13	Kelimandingju	134/150			Guposhanyeju	129/129
		Yinduyeju	139/139			Pixeju	134/134			Mangshanyeju	129/137
		Lihuaju	163/177			Katsuyamano	122/140	S85	4	Lihuaju	129/133
		Murcott	169/169			Gonggan	134/140			Guposhanchougan No5	125/125
S28	1	Yinduyeju	312/132			Wogan	134/158				
S73	2	Jianyemangshanyegan	345/345			Huangmeiren	128/158				
		Banyeshenggan	366/366			Aiyuan No38	128/150				

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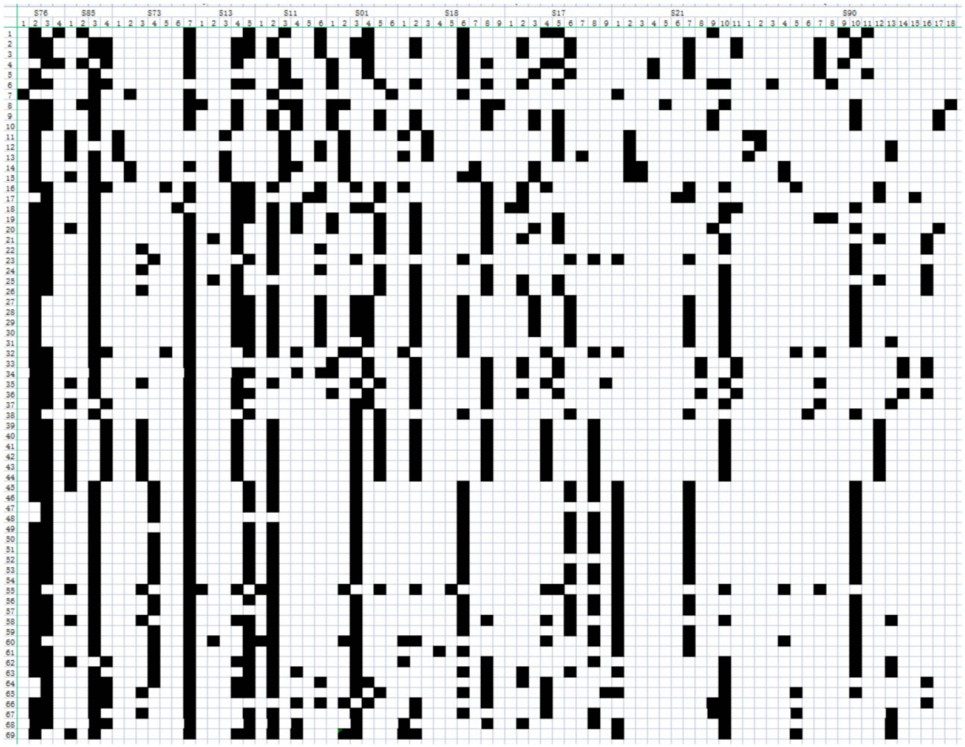

Figure 2. Fingerprint identity IDs of 69 *C.reticulata* SSR.

3.3.2. Varietal Discrimination Using Core Primer Combinations

A single core primer pair (selected from the 10 core primers) failed to fully discriminate all 69 mandarin accessions. Among the individual primers, S90 exhibited the highest discrimination efficiency, distinguishing 20 accessions with a discrimination rate of 28.98%. When all 10 core primer pairs were combined, 45 out of 69 accessions (65.2% discrimination rate) were successfully differentiated (Table 4). The remaining 24 indistinguishable accessions were clustered into six groups, reflecting their close genetic relationships—likely attributed to conserved genomic sequences and similar genetic backgrounds, which may result from common ancestry or artificial selection.

3.3.3. Molecular Identity Card Construction

Following the predefined coding rule (numeric codes 1–9 and uppercase letters for additional genotypes, with "0" for null alleles), the amplified fragment sizes of the 69 mandarin accessions were encoded using the 10 core primer pairs (Table 5). This resulted in the generation of 69 unique molecular identity cards for the tested mandarin accessions (Table 6). Among these, 45 molecular identity cards were distinct, corresponding to the 45 discriminable accessions, indicating that these accessions possess unique allelic profiles that can serve as diagnostic markers for their accurate authentication.

Table 4. Discrimination ability of 10 primer combinations.

Primer combination	Number of varieties identified	Differentiation rate(%)
S90	20	28.99
S90+S18	23	33.33
S90+S11	23	33.33
S90+S17	24	34.78
S90+S01	24	34.78
S90+S73	21	30.43

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S90+S21	21	30.43
S90+S85	22	31.88
S90+S11+S13	23	33.33
S90+S76+S11+S17+S13	24	34.78
Total	45	65.22

Table 5. Allele size ranges amplified by SSR primers and encoding standard.

Code	S76	S85	S73	S13	S11	S01	S18	S17	S21	S90
1	168/168	125/125	344/344	230/230	172/179	282/283	230/231	166/170	224/259	148/150
2	174/174	125/133	345/345	230/245	178/185	282/296	230/233	170/170	224/267	148/173
3	174/180	125/137	345/369	239/245	179/179	282/299	230/243	170/180	244/244	150/173
4	174/186	129/129	348/348	239/248	179/185	283/283	230/247	170/182	244/259	152/164
5	180/180	129/133	348/369	242/242	179/191	283/290	231/239	170/184	244/267	157/157
6	180/186	129/137	356/369	242/248	182/182	283/296	231/243	170/198	246/246	157/161
7		133/133	359/369	245/245	182/185	283/299	231/247	172/172	246/248	157/166
8		133/137	366/366	245/248	182/191	290/290	233/233	172/182	249/259	158/161
9			369/369	248/248	185/191	290/296	237/243	172/184	251/267	158/166
A				251/251	188/191	290/299	243/243	180/180	255/259	158/172
B						296/296	243/246	180/182	259/259	158/173
C						299/299	243/247	180/184	259/267	159/166
D						307/307	246/246	180/198	259/269	161/161
E							247/247	180/200	261/269	161/164
F							247/249	182/182	263/263	161/165
G								182/190	263/267	161/166
H								184/184	267/267	161/171
I								184/198	267/269	161/173
J								198/198	269/269	165/171
K										166/166
M										166/173
N										166/179
P										166/182
Q										166/202
R										172/172
S										172/177
T										172/179
U										173/173
V										176/179
W										179/179

Table 6. Molecular IDs for C. reticulata accessions based on SSR markers.

Germless name	Molecular ID	Germless name	Molecular ID
Guposhanyeju	44998BABFJ	Yingxinju	37980274EV
Xinganyeju	38985965DG	Pixeju	33970970HI
Daoxianyeju	38985965DG	Bendizao	279808AHBC
Mangshanyeju	669762CB8F	Penggan No79-2	33573A7DHR
Niuduyeju	279062A98H	Dong No13penggan	33573A7DHR
Hezhouyeju	38087374G4	Shi No18penggan	33573A7DHR
Yinduyeju	173A3DA030	Taitianpenggan	33573A7DHR
Lihuaju	359271F09Q	Xinshengxi No3penggan	33573A7DHR
Cengxisuanju	37974378FP	Wuhepenggan	33573A7DHR
Guangxihongpisuanju	37974378FP	Dafen No4	326938AI4K

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Germless name	Molecular ID	Germless name	Molecular ID
Guposhanchougan No2	2215642F61	Rinan No1	376938AI4K
Guposhanchougan No5	2110848F63	Dapu No5	576938A04K
Guposhanchougan No6	2215842G62	Miyamoto	576008AI4K
Yuanyemangshanyegan	273574D775	Miyagawa	379938AI4K
Jianyemangshanyegan	222564B775	Hashikawa	376938AI4K
Shagan	38785A43CA	Xingjin	376938AI4K
Biangan	5798ACE2AS	Yoshida	376938A04K
Banyeshenggan	37884971IK	Ichibun	376938AI4K
Huangpisuanju	3798437FHE	Yamasitabeni	376938AI4K
Hongpisuanju	32974378FP	Katsuyamano	2253175B56
Shatangju	37933C74HT	Shangye	376938AI4K
Zaoshushatangju	37575C70HN	Dajin No4	376038AI4K
Yamada	376938AI4K	Zaoxiang	325838CC5M
Bayueju	37575C70HN	Sakikubo	376938AI4K
Denglongju	37930C74HT	Jinzhixiang	3764151D47
Jinkuimiju	37573C74HN	Youliang	376938904K
Nanfengmiju1	27985969CK	Chunjian	3368384JHM
Nanfengmiju2	27985969CK	Nanxiang	376948C55M
Tezaoshumiju	27985969CK	Murcott	586859E3HW
Liuchengmiju	27985969CK	Gonggan	58583ACE59
Guijuyihao	27985B6HCM	Wogan	3809967AGW
Clementine	3879453D58	Huangmeiren	3759386DGB
Mingliutianju	37900274EV	Aiyuan No38	389948465U
Chuntianju	37989274EV	Mingrijian	229845105B
Guangxiju	32573A7EHD		

4. Discussion

Mandarin citrus (*Citrus reticulata*) has evolved diverse local accessions through long-term cultivation and breeding. Accessions derived from bud mutations or seedling selection often share highly similar genetic backgrounds, posing challenges for accurate identification. Molecular markers, which capture allelic variations, are therefore crucial for revealing genetic relationships among citrus germplasms. In this study, several accessions with known genetic origins were included: extra-early-maturing tangerine (bud sport of 'Nanfeng' tangerine), 'Miyakawa Bun' (bud sport selection of 'Miyagawa'), 'Miyagawa' (bud sport of Satsuma mandarin), 'Hashimoto' (bud sport of 'Matsuyama Satsuma'), 'Shiwen' and 'Shanxiahong' (bud sports of 'Miyagawa'), 'Mingliutianju' (bud sport of 'Chuntian' tangerine), 'Huacheng No.1' (seedling selection of sweet orange), 'Xinshengxi No.3 Ponkan' and 'Taitian Ponkan' (seedling selections of Ponkan), 'Dajin No.4' (seedling selection of Satsuma mandarin), 'Okitsu' (nucellar line of 'Miyagawa'), and the hybrid 'Tsunoka tangor' (cross of 'Kiyomi' × 'Okitsu'). Our results showed that germplasms with identical SSR banding patterns clustered together, reflecting genetic conservation in citrus, while divergent allelic variation sites indicated genetic differentiation. Notably, cluster analysis revealed co-grouping of certain wild and cultivated accessions that deviated from traditional taxonomic classifications, suggesting complex genetic affinities among mandarin germplasms.

4.1. Genetic Diversity of Mandarin Germplasms

Simple sequence repeat (SSR) markers are widely recognized as robust tools for assessing plant genetic variation and have been extensively applied in fruit tree genetic diversity studies, including pear [26], apple [27], and persimmon [28]. In this study, we combined SSR markers with fluorescent capillary electrophoresis to analyze the genetic diversity of 69 *C. reticulata* accessions. The mean Shannon's information index (I = 1.480) indicated substantial genetic diversity within the tested

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mandarin germplasms. For 16 loci, observed heterozygosity (Ho) ranged from 0.319 to 0.833, and expected heterozygosity (He) spanned 0.530 to 0.836—further confirming high genetic diversity among these accessions. Polymorphic information content (PIC), which quantifies polymorphism more accurately than allele number alone by integrating allele frequency, is a key indicator of marker utility. Eight loci in this study exhibited PIC values exceeding 0.65 (the threshold for high polymorphism), indicating their strong potential for citrus germplasm characterization. It should be noted that PIC values are material-dependent, as they vary with allele frequency differences across experimental samples. These diversity indices collectively reflect the magnitude of genetic variation, with higher values corresponding to increased heterozygosity—findings consistent with the rich genetic diversity of mandarins in China, a primary center of origin [4,5].

4.2. Insights from Cluster Analysis

The 69 mandarin accessions were clustered into three major groups, with several notable patterns:

1. 'Yinduyeju' formed a distinct single-accession group. Previous studies have debated its taxonomic status: Swingle [29] proposed it is a hybrid, while Yang et al. [30] clustered it with true citrons based on cpInDel markers, and Li et al. [31] suggested it is a primitive mandarin species. In contrast, 'Mangshanyeju' and 'Mangshan wild mandarin' did not cluster together, indicating a distant genetic relationship—consistent with the view that 'Mangshan wild mandarin' is more primitive than 'Mangshanyeju'. Additionally, Tachibana orange (native to Taiwan, China, and Japan) did not cluster with 'Yinduyeju' and was separated from other cultivated and wild accessions, which contradicts the findings of Xie et al. [32]. This discrepancy may be attributed to material errors during multi-location transfers, requiring further verification with authenticated germplasms.

2. 'Mangshanyeju' clustered with 'Nieduyeju' but separated from 'Guposhanyeju', indicating a closer genetic relationship between the former two. Liu et al. [33] identified 'Mangshanyeju' as the most ancient type among five wild mandarin types distributed in the Lingnan Mountains using SSR markers, and Zeng et al. [34] also considered it more primitive than Tachibana orange and 'Daoxianyeju'. Shi [1989] noted high similarity between 'Guposhanyeju' and 'Daoxianyeju', the latter of which is regarded as a progenitor of mandarins due to its distant genetic relationship with most cultivated accessions [34].

3. 'Guposhanchougan' clustered with 'Mangshanyegan' (both pointed-leaf and round-leaf types), consistent with the pollen morphology-based clustering results of Wu et al. [3]. 'Cenxisuanju', 'Guangxihongpisuanju', and 'Hongpisuanju' formed a distinct subgroup, separate from 'Hezhouyeju' and 'Huangpisuanju'—indicating genetic relatedness among the former three, which aligns with molecular marker-based clustering by Liu et al. [35]. 'Hezhouyeju' and 'Huangpisuanju' co-clustered, suggesting a potential genetic relationship that requires further validation via genomic analyses. In contrast, 'Shagan' and 'Biangan' did not cluster together, which contradicts Wu et al.'s [3] pollen morphology results, necessitating additional research to resolve this inconsistency.

4. Accessions with highly similar genetic backgrounds (e.g., bud sports) clustered closely, which is consistent with previous studies: 'Shatangju', early-ripening 'Shatangju', 'Bayueju', 'Denglongju', and 'Jinkui tangerine' co-grouped, matching Yan et al.'s [36] SRAP marker clustering; 'Chuntian tangerine', 'Mingliutianju', and 'Yingxinju' clustered with 'Biangan', while 'Shagan' and 'Gonggan' formed a separate subgroup; 'Guangxiju' clustered with Ponkan, and 'Wogan' grouped with 'W. Murcott'—all suggesting potential kinship.

5. Wild-cultivated germplasm relationships were also revealed: Group I contained Indian wild mandarin as a distinct lineage; Group II included wild wrinkled-skin mandarin and 'Mangshan wild mandarin', consistent with Wu et al. [3]; Group III comprised 'Guposhan wild Yuanju', 'Mangshan wild mandarin', 'Niedu wild mandarin', 'Shengshan wild mandarin', and the hybrid 'Tsunoka'—the latter two may carry wild genetic components. 'Hezhou wild mandarin' and 'Biangan' co-clustered with 'Shatangju' and Ponkan, demonstrating kinship. However, definitive cultivated-wild relationships require advanced genomic sequencing.

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Notably, Satsuma mandarin, Ponkan, and Shatangju accessions—primarily derived from bud sports or nucellar lines—clustered closely due to their genetic proximity. Bud sports arise from minor genomic alterations, which are difficult to detect using conventional molecular markers. Transposons (autonomous mobile DNA sequences) that transpose and insert adjacent to or within genes are a primary driver of bud sport formation [37]. Emerging techniques show promise for bud sport discrimination: Ke et al. [38] successfully distinguished citrus bud sports from conventional varieties using transposon display (TD) technology; Zhu et al. [39] established an efficient identification system using Target SSR-seq (an NGS-based SSR genotyping method), authenticating 60 citrus cultivars. While this study did not achieve complete differentiation of bud sports, the precise amplified fragment sizes obtained via SSR markers combined with fluorescent capillary electrophoresis provide a foundational reference for subsequent bud sport-focused research.

4.3. Value of DNA Fingerprints and Molecular Identity Cards

DNA fingerprinting, which visualizes PCR-amplified molecular markers, is widely adopted for cultivar identification due to its efficiency, accuracy, cost-effectiveness, and reproducibility. Previous studies have established citrus fingerprint databases using SSR markers: Li et al. [22] screened 362 SSR primer pairs to identify 21 highly polymorphic core primers, constructing a fingerprint database for 500 accessions spanning the Papeda and *Citrus* genera (including citron, lemon, lime, mandarin, and sweet orange); Lei et al. [21] selected 12 diagnostic primers from 200 SSR pairs to build fingerprints for 70 cultivated citrus accessions (oranges, pomelos, ponkan, tangors). Expanding on these efforts, our study incorporated both wild and cultivated mandarin accessions, developing DNA fingerprints for 69 germplasms using SSR markers combined with fluorescent capillary electrophoresis. To enhance visual discrimination, we converted fingerprint data into intuitive binary matrices in spreadsheets—facilitating rapid accession comparison.

Molecular identity (ID) cards, which transform molecular data into unique alphanumeric codes, have been widely applied in crop cultivar authentication, initially in rice and soybean, and later extended to fruit trees such as apple and pear. Lei et al. [14] used polyacrylamide gel electrophoresis (PAGE) to convert gel banding patterns into binary (0/1) matrices, generating cultivar-specific fingerprint codes by concatenating alphabetically coded primer sequences. However, conventional PAGE only approximates DNA fragment sizes via molecular weight marker comparison, lacking precision. In contrast, fluorescence-labeled SSR capillary electrophoresis enables precise fragment sizing with superior accuracy, sensitivity, and efficiency—addressing the limitations of PAGE.

In this study, we used fluorescent capillary electrophoresis to obtain exact fragment molecular weights, coded amplicons in ascending order using Arabic numerals and uppercase letters, and concatenated these codes to create unique molecular IDs for the 69 mandarin accessions. Similar approaches have been successfully applied in other citrus-related studies: Wu et al. [15] constructed 22 pummelo molecular IDs via fluorescent SSR capillary electrophoresis; Gao et al. [40] developed scannable QR-code IDs for 314 apple accessions using 6 SSR markers; Tang et al. [41] built molecular IDs for 145 mango germplasms with 12 fluorescent SSRs. To optimize cost-efficiency, we implemented a tiered screening strategy: agarose gel electrophoresis for preliminary amplification validation, PAGE for polymorphism assessment, and fluorescent capillary electrophoresis for precise sizing—yielding 225 genotypes and 139 alleles. While the capillary electrophoresis-based molecular IDs require further validation for direct citrus cultivar authentication, they represent a standardized tool for germplasm discrimination, variety protection, and breeding.

4.4. Limitations and Future Perspectives

This study successfully constructed DNA fingerprints and molecular IDs for 69 mandarin accessions, with 45 (65.22%) exhibiting unique IDs distinguishable using 10 core primer pairs. The remaining 24 undifferentiated accessions formed six clusters of genetically homologous germplasms, which require further analysis. A key limitation is the inability to fully differentiate bud sports, which

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is attributed to the minor genomic alterations underlying bud sport formation and the resolution limits of conventional SSR markers.

Future work will address these limitations by: (1) expanding the germplasm scope to include more wild and cultivated mandarin accessions, establishing a comprehensive molecular ID database; (2) integrating advanced technologies (e.g., transposon display, Target SSR-seq, and whole-genome sequencing) to improve bud sport discrimination; (3) validating the developed molecular IDs in multi-environment and multi-year trials to enhance their reliability for practical cultivar authentication; and (4) combining molecular IDs with phenotypic traits to resolve citrus nomenclature conflicts and clarify genetic relationships. These efforts will advance mandarin germplasm management, support intellectual property protection, and promote the sustainable development of the citrus industry.

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