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Genetic Diversity Analysis of Guangxi Kumquat (*Fortunella* Swing) Germplasm Using SRAP Markers

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Abstract: In order to understand the genetic diversity of germplasm resources of kumquats in Guangxi, 14 kumquat germplasm resources in Guangxi and 12 accessions from other provinces were analyzed by using SRAP markers. In total, 19 primer pairs with high stability, good reproducibility, and high polymorphism were chosen for analysis of all 26 kumquat genotypes. Among the 104 amplified bands, 90 (86.54%) were polymorphic. SRAP markers were analyzed by employing Principal Coordinate Analysis, Population Structure Analysis, and Hierarchical Cluster Analysis (UPGMA). The classification results showed that the 26 kumquat germplasm resources could be divided into 5 groups, including cultivated kumquat, intergeneric hybrid, wild kumquat from other provinces, wild kumquat, and hybrid kumquat from Guangxi. The Guangxi kumquat germplasm had high genetic diversity, and were clearly divided into three groups: cultivated kumquat, wild kumquat, and hybrid kumquat. Additionally, the eight cultivated kumquat varieties in Guangxi were further divided into two subgroups. Wild kumquat in Guangxi or in other provinces belonged to different groups; meanwhile, the Guangxi kumquat hybrid formed an independent group, thus indicating that Guangxi wild kumquat and hybrid kumquat possess certain specificity, or they possibly belong to different species. Among the tested 26 kumquat accessions, 23 unique genotype-specific SRAP markers were detected for 14 kumquat genotypes, which were positively identified. For the remaining 12 accessions without genotype-specific markers, they were distinguished by various combinations of markers. These results may have certain importance for kumquat genetic research and cultivar selection.

Keywords: *Fortunella* spp.; molecular marker; principal coordinate; population structure; hierarchical cluster; specific markers

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1. Introduction

Guangxi is located in the southwest of China and has a warm subtropical monsoon climate and diverse topography, making it a suitable area for growing citrus since ancient times [1]. With its abundant citrus genetic resources and long cultivation history, Guangxi occupies the number one position in citrus production in China. Kumquat (*Fortunella* Swing) is a genus close to *Citrus* but with distinctive fruit characteristics [2]. Kumquat fruit is rich in flavonoids, and the main phenolic compounds are C-glycoside flavonoids, which are different from those in citrus fruits [3]. *F. margarita* peel was reported to be a rich source of potentially bioactive polyphenols [4]. β-cryptoxanthin (BCX) was also identified as an active kumquat component with an NK cell-activating effect, and *R*-limonene as an active component that mediates not only the anti-stress effect but also NK cell activation by oral administration [5].

Kumquat serves as a key industry in Yangshuo and Rongan counties in Guangxi. The primary cultivar is 'Rongan' kumquat, which has given rise to a series of new varieties [6–9].

Moreover, Guangxi possesses *F. hindsii* and some natural hybrids. Li et al. conducted ploidy analysis and SSR identification of 'Gui Shanjingan' (*F. hindsii* from Guangxi). The results indicated that there was a significant genetic difference between 'Gui Shanjingan' and the *F. hindsii* genotypes from other provinces, and it seemed to be a unique wild kumquat [10]. Additionally, Huang et al. collected Shanju (a kumquat genotype in Guangxi) resources at the border between China and Vietnam, and the genetic analysis results suggested that Shanju could be a new kumquat variety or species. Seeds of Shanju are mono-embryonic and its seedlings have a short juvenile period; thus, it could be utilized as an effective breeding model plant for kumquat [11].

There are different opinions about the classification of kumquat. In The Chinese Record of Fruit Tree—Citrus Volume, kumquat is considered an independent genus, which includes five species and one intergeneric hybrid. In detail, the species are Longleaf kumquat (*F. polyandra*), Hongkang kumquat or golden bean (*F. hindsii*), Luofu (*F. margarita*), Luowen (*F. japonica*), and Jindan (*F. crassifolia*), and the intergenus hybrid genotypes such as Changshou kumquat, Sijiju, etc. [2]. In Flora of China (1997 version), five kumquat species were also recorded with some modified scientific names, including *F. bawangica*, *F. hindsii*, *F. japonica*, *F. margarita*, and *F. venosa*. However, in the 2020 version of the same Flora of China, kumquat was incorporated into *Citrus*, and the species *F. hindsii*, *F. japonica*, *F. margarita*, and *F. venosa* were united as one species—*Citrus japonica* Thunb. However, the Bawang kumquat remained as the previous *F. bawangica* Huang [12]. Such confusion in kumquat classification leads to an obstacle for kumquat germplasm studies. It has also been unclear as to the evolutionary relationship between cultivated and wild kumquats and whether *F. crassifolia* is a pure species or a hybrid.

There have been some reports on the origin and classification of kumquat using molecular markers. Cheng et al. used cp-SSR markers to prove that Sijiju is a hybrid of kumquat (maternal) and tangerine (paternal) [13]. By using RAPD and chloroplast CAPs molecular marker analysis, Yasuda et al. believed that Luowen, Luofu, and Jindan should be one group or species [14]. The chloroplast genome analysis results of Wang et al. supported the incorporation of kumquat into *Citrus*, but there should be three species, namely *C. venosa*, *C. hindsii*, and *C. japonica* [15]. Zhu et al. analyzed the genetic diversity and phylogeny of 38 kumquat accessions using 46 nuclear SSR and 5 chloroplast loci, and their results suggested that kumquat contained 2 major populations: cultivated kumquat [Luofu (*F. margarita*), Luowen (*F. japonica*), and Jindan (*F. crassifolia*)] and wild kumquat (Hongkong wild kumquat). They further indicated that Luowen originated from the cross or backcross between Luofu and Jindan, but all three deserved the status of "species" [16]. SSR clustering results supported the position of Changshou kumagat as a species [17].

Sequence-related amplification polymorphism (SRAP) employs PCR to detect polymorphisms in the lengths of introns, promoters, or spacers among different individuals and species. Due to its simplicity and effectiveness, SRAP has found extensive application in the analysis of genetic diversity, construction of genetic maps, mapping of crucial traits, and cloning of related genes in plants, including grapes, oil palms, plums, and mangoes [18–21].

In this study, we employed SRAP markers to assess the genetic diversity of Guangxi kumquat germplasm resources and to develop specific markers for germplasm identification, thus providing more knowledge for kumquat classification, germplasm conservation, and better utilization of kumquat resources in Guangxi.

2. Materials and Methods

2.1. Materials

Twenty-six kumquat genotypes (Table 1) were collected from Guangxi Citrus Germplasm Repository at Guangxi Academy of Specialty Crops and the National Citrus Germplasm Repository at Citrus Research Institute, Southwest University (Chongqing). For each kumquat accession, 5–10 plants were propagated by graft on trifoliate orange rootstock and grown in a greenhouse. Young leaves were sampled from the plants of each genotype and mixed for SRAP analysis.

Table 1. The tested kumquat genotypes.

Code	Abbreviation	Genotype Name	Scientific Name	Possible Origin
1	NB jindan	Ningbo jindan	*F. crassifolia*	Ningbo, Zhejiang
2	Daguojindou	Daguojindou	*F. hindsii*	Citrus Research Institute, SWU/CAAS
3	Jinganzazhong	Guangxi natural kumquat hybrid	*Citrus* × *Fortunella*	Hezhou, Guangxi
4	WZ luofu	Wenzhou luofu	*F. margarita*	Wenzhou, Zhejiang
5	NB luofu	Ningbo luofu	*F. margarita*	Ningbo, Zhejiang
6	WZ jingdan	Wenzhou jingdan	*F. crassifolia*	Wenzhou, Zhejiang
7	Sijiju	Sijiju	*Citrus* × *Fortunella*	Citrus Research Institute, SWU/CAAS
8	Wenzhouju	Wenzhouju (kumquat hybrid)	*Citrus* × *Fortunella*	Wenzhou, Zhejiang
9	Shouxingju	Shouxingju (kumquat hybrid)	*Citrus* × *Fortunella*	Citrus Research Institute, SWU/CAAS
10	Dajindou	Dajindou	*F. hindsii*	Citrus Research Institute, SWU/CAAS
11	NB luowen	Ningbo luowen	*F. japonica*	Ningbo, Zhejiang
12	RA jingan	Rongan jingan	*F. crassifolia*	Liuzhou, Guangxi
13	FY jingan	Fuyuan jingan	*F. crassifolia*	Liuzhou, Guangxi
14	CM jingan	Cuimi jingan	*F. crassifolia*	Liuzhou, Guangxi
15	Guijingan1	Guijingan No.1	*F. crassifolia*	Yangshuo, Guangxi
16	Guijingan2	Guijingan No.2	*F. crassifolia*	Yangshuo, Guangxi
17	YS jingan	Yangshuo jingan	*F. crassifolia*	Yangshuo, Guangxi
18	F15-1	F15-1	*F. crassifolia*	Liuzhou, Guangxi
19	Shanjingan	Hunan Shanjingan	*F. hindsii*	Changsha, Hunan
20	LY jingan	Liuyang jingan	*F. crassifolia*	Changsha, Hunan
21	HP jingan	Huapi jingan	*F. crassifolia*	Liuzhou, Guangxi
22	FC-1	Guangxi wild kumquat FC-1	*Fortunella* sp.	Fangchenggang, Guangxi
23	FC-2	Guangxi wild kumquat FC-2	*Fortunella* sp.	Fangchenggang, Guangxi
24	FC-3	Guangxi wild kumquat FC-3	*Fortunella* sp.	Fangchenggang, Guangxi
25	FC-4	Guangxi wild kumquat FC-4	*Fortunella* sp.	Fangchenggang, Guangxi
26	FC-5	Guangxi wild kumquat FC-5	*Fortunella* sp.	Fangchenggang, Guangxi

Note: SWU: Southwest University; CAAS: Chinese Academy of Agricultural Sciences.

2.2. DNA Isolation

The genomic DNA from the leaves of 26 kumquat genotypes was isolated by using an improved CTAB protocol [22]. DNA concentration was determined with the Nanodrop 2000 test. The qualified DNA had a ratio of 260/280 above 1.8. Then, the concentration and quality of the DNA were confirmed by 1% agarose gel electrophoresis.

2.3. PCR Amplification for SRAP Markers

Referring to the primer combinations reported by Zhang et al. [23], 4 kumquat varieties (Daguojindou, NB luofu, RA jingan, and HP jingan) were used for screening of suitable primers among 420 primer pairs (Table 2). In total, 19 primer pairs (Table 3) with high stability, good reproducibility, and high polymorphism were chosen for further analysis of all 26 kumquat genotypes.

The SRAP–PCR reaction was carried out as described by Xu et al. [24] with slight modification. Specifically, 0.12 mM dNTP, 0.2 μM primers, 5 U Taq DNA polymerase, 2 μL 10× Taq Buffer (containing 1.6 mM Mg^{2+}), and 50 ng of template DNA were added to a total of 20 μL SRAP-PCR reaction solution. The PCR amplification procedure consisted of an initial denaturation step at 94 °C for 5 min, followed by five cycles of 94 °C for 60 s, 35 °C for 60 s, and 72 °C for 2 min, and then 35 cycles of 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s. A final extension was performed at 72 °C for 8 min. PCR products were stored at 4 °C. All PCR amplifications were repeated at least twice.

2.4. Agarose Gel Electrophoresis

The SRAP-PCR products were separated on a 2% agarose gel by electrophoresis for 1.5 to 2 h, subsequently stained with 4S Green Nucleic Acid Stain (Perfemiker, Shanghai, China), and then photographed using an imager [24].

Table 2. All primer pairs used for suitable SRAP primer screening in this study.

Forward Primer		Reverse Primer	
ME Primer Code	**Primer Sequence (5′-3′)**	**EM Primer Code**	**Sequence (5′-3′)**
ME1	TGAGTCCAAACCGGAAA	EM1	GACTGCGTACGAATTAAC
ME2	TGAGTCCAAACCGGAAC	EM2	GACTGCGTACGAATTAAT
ME3	TGAGTCCAAACCGGAAG	EM3	GACTGCGTACGAATTACG
ME4	TGAGTCCAAACCGGAAT	EM4	GACTGCGTACGAATTAGC
ME5	TGAGTCCAAACCGGACA	EM5	GACTGCGTACGAATTATG
ME6	TGAGTCCAAACCGGACC	EM6	GACTGCGTACGAATTCAA
ME7	TGAGTCCAAACCGGACG	EM7	GACTGCGTACGAATTCAC
ME8	TGAGTCCAAACCGGACT	EM8	GACTGCGTACGAATTCAG
ME9	TGAGTCCAAACCGGAGA	EM9	GACTGCGTACGAATTCAT
ME10	TGAGTCCAAACCGGAGC	EM10	GACTGCGTACGAATTCCA
ME11	TGAGTCCAAACCGGAGG	EM11	GACTGCGTACGAATTCGA
ME12	TGAGTCCAAACCGGATA	EM12	GACTGCGTACGAATTCGG
ME13	TGAGTCCAAACCGGTAA	EM13	GACTGCGTACGAATTCTA
ME14	TGAGTCCAAACCGGTAG	EM14	GACTGCGTACGAATTCTC
ME15	TGAGTCCAAACCGGTCA	EM15	GACTGCGTACGAATTCTG
ME16	TGAGTCCAAACCGGTCC	EM16	GACTGCGTACGAATTCTT
ME17	TGAGTCCAAACCGGTGC	EM17	GACTGCGTACGAATTGAT
ME18	TGAGTCCAAACCGGTGT	EM18	GACTGCGTACGAATTGCA
ME19	TGAGTCCAAACCGGTTA	EM19	GACTGCGTACGAATTGGT
ME20	TGAGTCCAAACCGGTTG	EM20	GACTGCGTACGAATTGTC
		EM21	GACTGCGTACGAATTTAG
		EM22	GACTGCGTACGAATTTCG
		EM23	GACTGCGTACGAATTTGA
		EM24	GACTGCGTACGAATTTGC

Note: Each ME primer is paired with any EM primer to form a primer pair.

Table 3. Analysis of the polymorphisms detected using 19 chosen SRAP primers.

No.	Primer Pair Code	Amplified Bands	Polymorphic Bands	Polymorphic Rate (%)
1	Me1Em15	6	4	66.67
2	Me1Em22	4	4	100
3	Me1Em23	6	6	100
4	Me2Em17	6	6	100
5	Me9Em23	7	4	57.14
6	Me2Em21	6	5	83.33
7	Me10Em7	10	8	80
8	Me4Em7	1	1	100
9	Me4Em12	10	8	80
10	Me3Em17	4	4	100
11	Me4Em17	4	4	100
12	Me11Em21	2	2	100
13	Me10Em13	4	4	100
14	Me14Em12	6	6	100
15	Me16Em19	4	2	50
16	Me7Em4	10	8	80
17	Me20Em2	7	7	100
18	Me17Em2	2	2	100
19	Me18Em22	5	5	100
Sum/Average		104/5.47	90/4.74	86.54

2.5. Parameters Used for Analysis of SRAP Markers

Bands with identical mobility among 26 kumquat genotypes, amplified with SRAP primers, were scored as "0" (absence of SRAP) and those that were polymorphic as "1" (presence of SRAP), resulting in the construction of a binary sequence matrix of "0, 1" [20]. Principal coordinate analysis (PCoA) was used to construct a biplot using PAST

3.11 software. Jaccard similarity coefficients were used to examine data from SRAP markers [25]. The population Structure analysis was performed using Structure 2.3.4 software and the optimal K value was determined using the ΔK values in the Structure Harvester analysis method [26,27]. The Unweighted Pair-Group Method with Arithmetic Mean Algorithm (UPGMA) in PAST 3.11 software was utilized to create phylogenetic trees [28,29]. Genotype-specific markers were searched through all the SRAP markers, which were used to identify kumquat accessions.

3. Results and Analysis

3.1. Polymorphism Analysis Using SRAP Markers

Out of the 420 pairs of primers screened, 19 SRAP markers were selected, as they yielded clear and bright bands. Figure 1 shows the PCR amplification products with SRAP primers ME20 + EM2. These primers were used to genotype 26 kumquat germplasm resources and 104 bands were amplified in total, with an average of 5.47 bands per primer pair. Among these bands, 90 were polymorphic. The percentage of polymorphism of each primer was 50–100%, and the average percentage of polymorphism was 86.54% (Table 3).

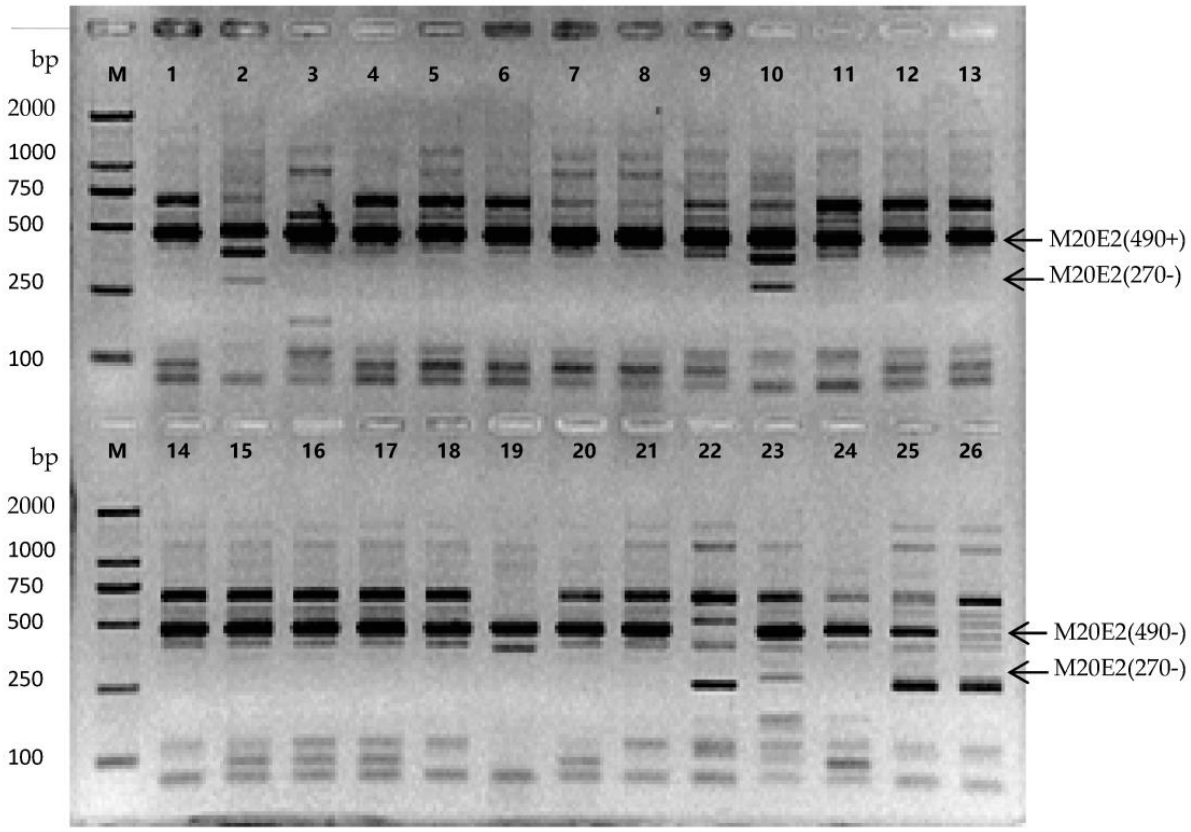

Figure 1. PCR amplification products with the SRAP primer pair ME20 + EM2. Note: 1–26 indicate the 26 kumquat genotypes listed in Table 1; M: DNA size ladder DL2000. Examples of markers: M20E2(490−): absence of a band as a common specific marker of FC-1 and FC-5; M20E2(270+): presence of a band as a common specific marker of FC-2 and Daguojindou.

3.2. Principal Coordinate Analysis

Principal Coordinate Analysis (PCoA) was performed on the data generated by the amplification of kumquat genomic DNA using 19 SRAP primer combinations. Coord. 1 represented 34.80% of the genetic variation in these samples, and Coord. 2 covered 19.25% of the genetic variation. The obtained eigenvalues indicated that the first two coordinates provided a good summary of the data, as they explained 54.05% of the total variability (Table 4).

Table 4. Eigenvalues of Principal Coordinate Analysis (PCoA).

Axis	Eigenvalue	Cumulative Eigenvalue	Percent (%)	Cumulative (%)
1	0.53	0.53	34.80	34.80
2	0.29	0.83	19.25	54.05
3	0.19	1.02	12.74	66.78
4	0.17	1.19	10.90	77.68
5	0.09	1.28	5.67	83.35
6	0.08	1.36	5.38	88.72
7	0.03	1.39	2.10	90.82
8	0.02	1.41	1.61	92.43
9	0.02	1.43	1.34	93.76
10	0.02	1.45	1.01	94.78
11	0.01	1.46	0.78	95.56
12	0.01	1.47	0.52	96.08
13	0.00	1.47	0.24	96.32
14	0.00	1.48	0.11	96.42
15	0.00	1.48	0.00	96.43
16	0.00	1.48	0.00	96.43

The biplot of PC1 and PC2 showed the 26 kumquats' grouping (Figure 2). On the PC1, 26 Kumquat accessions were divided into two main categories. The first group included Daguojindou, Dajindou, Shanjingan, FC-1, FC-2, FC-3, FC-4, FC-5, and Jinganzazhong, which belonged to the wild kumquat germplasm. The second group combined the intergenus hybrids (Wenzhouju, Sijiju, and Shouxingju) and all cultivated kumquat varieties. On the PC2, the two groups could be further divided into five subgroups. The first group was classified into three subgroups, namely Hunan wild kumquat (Daguojindou, Dajindou, and Shanjingan), Guangxi wild kumquat (FC-1, FC-2, FC-3, FC-4, and FC-5), and Jinganzazhong. The second one covered the subgroup of intergenus hybrids (Wenzhouju, Sijiju, and Shouxingju) and that of cultivated kumquat varieties. However, the cultivar NB luofu remained a certain distance from the others.

According to the combination of PC1 and PC2, 26 kumquat genotypes could be divided into 5 groups. The first group was wild kumquat (*F. hindisii*), comprising Daguojindou, Dajindou, and Shanjingan from Hunan province. The second one was occupied by Guangxi wild kumquats FC-1, FC-2, FC-3, FC-4, and FC-5. The third one contained 14 kumquat cultivars collected from different locations. The fourth one was the intergeneric hybrids (Wenzhouju, Sijiju, and Shouxingju). Finally, the fifth one was Jinganzazhong, the wild hybrid from Gupo Mountain in Hezhou, Guangxi.

3.3. Population Structure Analysis

The admixture simulation model was used to assess the kumquat clustering types by screening 19 SRAP primer combinations on the 26 genotypes. The cluster range was evaluated from K = 1 to K = 10. The output results showed a sharp peak with no ambiguity, indicating the highest delta K value at K = 2. There was a second sharp peak at K = 5 (Figure 3). Furthermore, the Bayesian bar graph was used to construct the graph for the admixture model. The accessions were grouped in subgroup clusters with >70% probability of membership fractions.

At K = 2, 11 out of 26 kumquats formed subpopulation I (red color, representing 42.3% of the total number of accessions), and 13 went into subpopulation II (green color, representing 50.0%) (Figure 4). Group I mainly contained wild kumquats; Group II included most cultivated accessions.

At K = 5, 13 kumquat genotypes, all cultivated varieties, gathered in subpopulation I (red color). The Guangxi wild hybrid Jinganzazhong solely occupied subpopulation II (green color). The three intergenus hybrids (Wenzhouju, Sijiju, and Shouxingju) were grouped in subpopulation III (blue color). The three wild kumquats (*F. hindisii*) from Hunan (Daguojindou, Dajindou, and Shanjingan) were grouped in subpopulation IV (yellow color).

Finally, the five wild kumquat genotypes from Guangxi (FC-1, FC-2, FC-3, FC-4, and FC-5) formed the subpopulation V (purple). Strangely, NB luofu did not fall in any group but had the mixed four groups' genetic background (Figure 5).

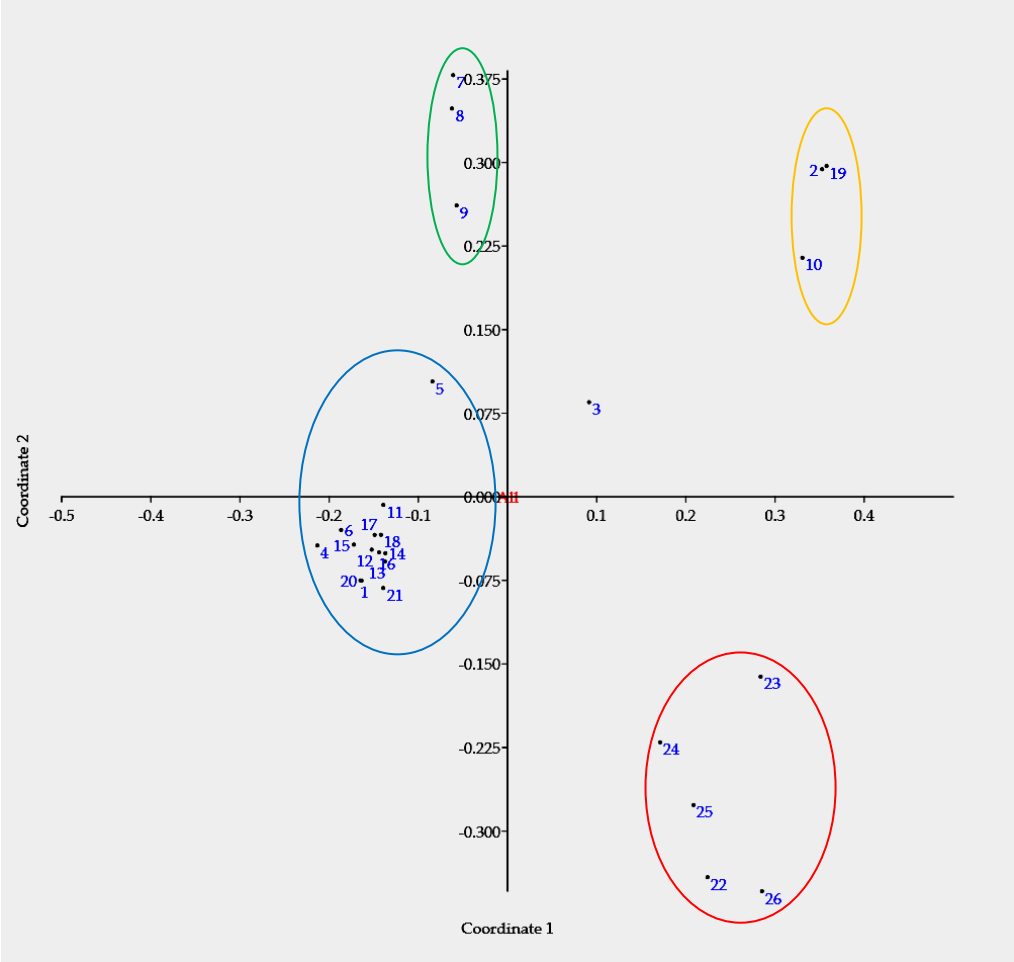

Figure 2. Biplot analysis of kumquats' diversity as inferred from SRAP markers. Note: The numbers are genotype codes listed in Table 1.

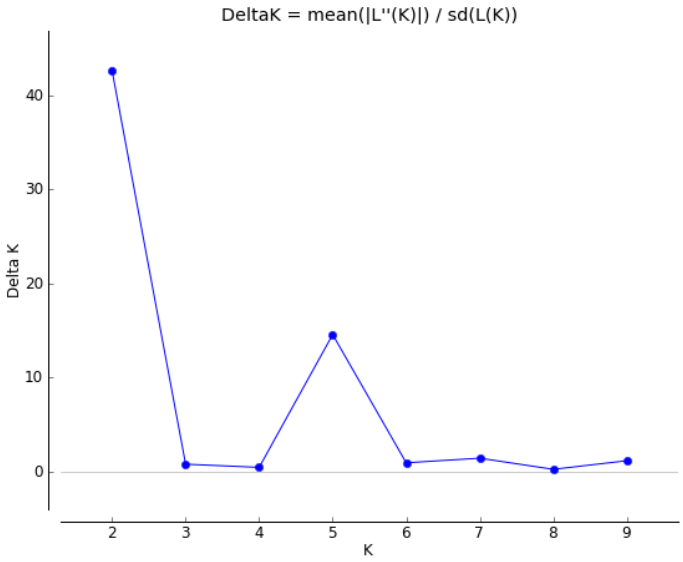

Figure 3. The number of K clusters (1–10) generated from nineteen SRAP primer combinations.

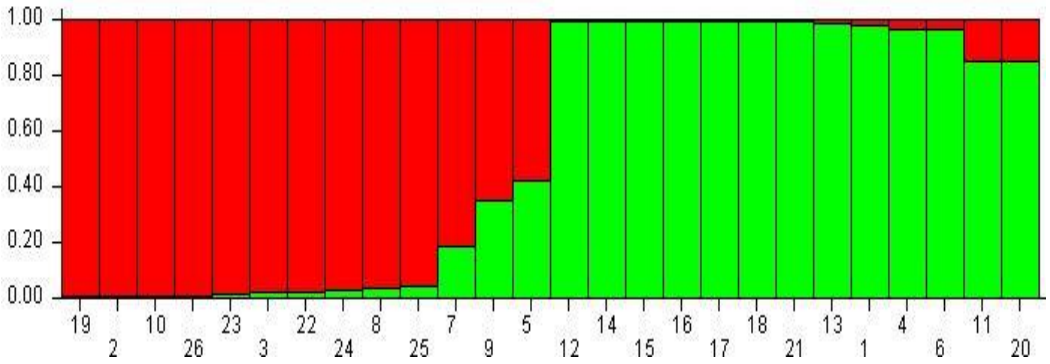

Figure 4. Population structure analysis at K = 2 using SRAP marker data from 26 kumquat genotypes. Note: The genotype codes are listed in Table 1.

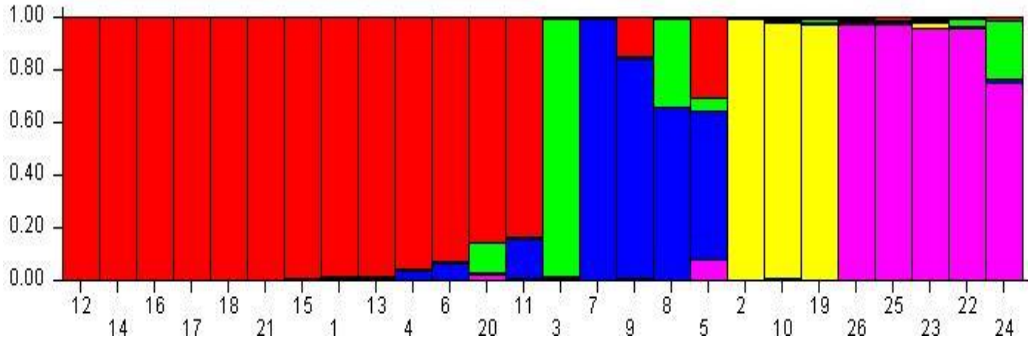

Figure 5. Structure analysis at K = 5 using SRAP marker data from 26 kumquat genotypes. Note: The genotype codes are listed in Table 1.

3.4. Hierarchical Cluster Analysis (HCA)

A cluster analysis was carried out using the Jaccard coefficient by the UPGMA method based on the Genetic similarity coefficients (Table 5). In the dendrogram, the kumquat genotypes were clustered into five groups (Figure 6). Group one (G1) was the largest, covering 14 kumquat cultivars collected from different locations. This group was further subdivided into four subgroups. Subgroup one contained five cultivars, Rongan jingan and its bud mutation varieties (FY jingan, Guijingan1, Guijingan2, and YS jingan), cultivated in Guangxi. HP jingan, a mutant variety of RA jingan, together with its mutants F15-1 and CM jingan (also from Guangxi), formed Subgroup two. The cultivars from Zhejing (NB jindan, WZ luofu, WZ jingdan, and NB luowen) and that from Hunan (LY jingan) gathered in Subgroup three; NB luofu from Zhejiang occupied a single subgroup. Group two (G2) comprised Wenzhouju, Sijiju, and Shouxingju, which are all intergenus hybrids. Group three (G3) contained FC-1, FC-2, FC-3, FC-4, and FC-5; these are wild kumquats collected from Guangxi. Group four involved Daguojindou, Dajindou, and Shanjingan, which are wild kumquats (*F. hindisii*) from Hunan province. The single genotype, Jinganzazhong from Gupo Mountain in Hezhou, Guangxi, clustered in the last group.

3.5. Screening of Genotype-Specific Markers and Identification od Kumquat Accessions

Looking through the SRAP markers, some unique kumquat genotype-specific markers (a band was present/absent only in one genotype but not in others) were detected. Among the 26 tested kumquat accessions, 14 genotypes presented 23 unique specific markers. Jinganzazhong, the kumquat hybrid from Guangxi, had four unique markers, NB luofu from Zhejiang had three, NB jindan, WZ jingdan, and Dajindou each had two, and the remaining had one for each (Table 6). NB jindan and WZ jingdan were the only cultivated varieties that had unique specific markers.

Table 5. Genetic similarity coefficients based on the SRAP markers of all the tested genotypes.

No.	Germplasm	1. NB jindan	2. Daguo jindou	3. Jingan zazhong	4. WZ luofu	5. NB luofu	6. WZ jingdan	7. Sijiju	8. Wen zhouju	9. Shou xingju	10. Da-jindou	11. NB luowen	12. RA jingan	13. FY jingan	14. CM jingan	15. Gui jingan1	16. Gui jingan2	17. YS jingan	18. F15-1	19. Shan jingan	20. LY jingan	21. HP jingan	22. FC14-1	23. FC14-2	24. FC14-3	25. FC14-4	26. FC14-5
1	**NB jindan**	1.000																									
2	**Daguojindou**	0.634	1.000																								
3	**Jinganzazhong**	0.634	0.604	1.00																							
4	**WZ luofu**	0.941	0.634	0.594	1.000																						
5	**NB luofu**	0.812	0.6436	0.584	0.851	1.000																					
6	**WZ jingdan**	0.941	0.634	0.653	0.921	0.812	1.000																				
7	**Sijiju**	0.762	0.673	0.634	0.762	0.851	0.782	1.000																			
8	**Wenzhouju**	0.703	0.594	0.584	0.703	0.772	0.703	0.871	1.000																		
9	**Shouxingju**	0.822	0.673	0.673	0.782	0.831	0.822	0.941	0.851	1.000																	
10	**Dajindou**	0.663	0.931	0.594	0.644	0.673	0.663	0.663	0.584	0.703	1.000																
11	**NB luowen**	0.911	0.663	0.644	0.911	0.822	0.911	0.772	0.723	0.832	0.693	1.000															
12	**RA jingan**	0.960	0.653	0.653	0.941	0.812	0.980	0.762	0.703	0.822	0.683	0.931	1.000														
13	**FY jingan**	0.950	0.663	0.663	0.931	0.802	0.970	0.752	0.693	0.812	0.693	0.921	0.990	1.000													
14	**CM jingan**	0.941	0.673	0.653	0.921	0.792	0.960	0.743	0.683	0.802	0.703	0.931	0.980	0.970	1.000												
15	**Guijingan1**	0.950	0.644	0.644	0.950	0.822	0.970	0.772	0.693	0.812	0.673	0.921	0.990	0.980	0.970	1.000											
16	**Guijingan2**	0.970	0.644	0.663	0.931	0.802	0.970	0.772	0.712	0.832	0.673	0.921	0.990	0.980	0.970	0.980	1.000										
17	**YS jingan**	0.960	0.653	0.653	0.941	0.792	0.960	0.782	0.723	0.822	0.663	0.911	0.980	0.970	0.960	0.970	0.990	1.000									
18	**F15-1**	0.931	0.683	0.644	0.931	0.782	0.950	0.752	0.693	0.792	0.693	0.921	0.970	0.960	0.990	0.960	0.960	0.970	1.000								
19	**Shanjingan**	0.644	0.911	0.594	0.644	0.653	0.624	0.683	0.614	0.683	0.881	0.653	0.644	0.653	0.663	0.634	0.653	0.663	0.673	1.000							
20	**LY jingan**	0.941	0.634	0.634	0.921	0.772	0.901	0.743	0.703	0.782	0.644	0.871	0.921	0.911	0.901	0.911	0.931	0.941	0.911	0.644	1.000						
21	**HP jingan**	0.960	0.653	0.653	0.921	0.792	0.941	0.743	0.693	0.802	0.683	0.931	0.960	0.950	0.980	0.950	0.970	0.960	0.970	0.663	0.921	1.000					
22	**FC14-1**	0.683	0.634	0.574	0.644	0.634	0.683	0.584	0.525	0.644	0.663	0.693	0.703	0.713	0.703	0.693	0.713	0.703	0.693	0.644	0.6434	0.703	1.000				
23	**FC14-2**	0.703	0.713	0.554	0.663	0.693	0.683	0.683	0.614	0.723	0.743	0.713	0.703	0.713	0.703	0.693	0.713	0.703	0.693	0.723	0.663	0.703	0.822	1.000			
24	**FC14-3**	0.693	0.644	0.683	0.673	0.743	0.713	0.653	0.604	0.693	0.653	0.723	0.713	0.723	0.713	0.703	0.703	0.693	0.703	0.634	0.673	0.713	0.713	0.772	1.000		
25	**FC14-4**	0.762	0.693	0.614	0.723	0.733	0.723	0.663	0.614	0.683	0.723	0.733	0.743	0.733	0.743	0.733	0.752	0.743	0.733	0.703	0.762	0.762	0.802	0.822	0.772	1.000	
26	**FC14-5**	0.683	0.653	0.594	0.663	0.673	0.644	0.604	0.545	0.624	0.683	0.653	0.663	0.673	0.663	0.653	0.673	0.683	0.673	0.663	0.703	0.683	0.762	0.782	0.733	0.861	1.000

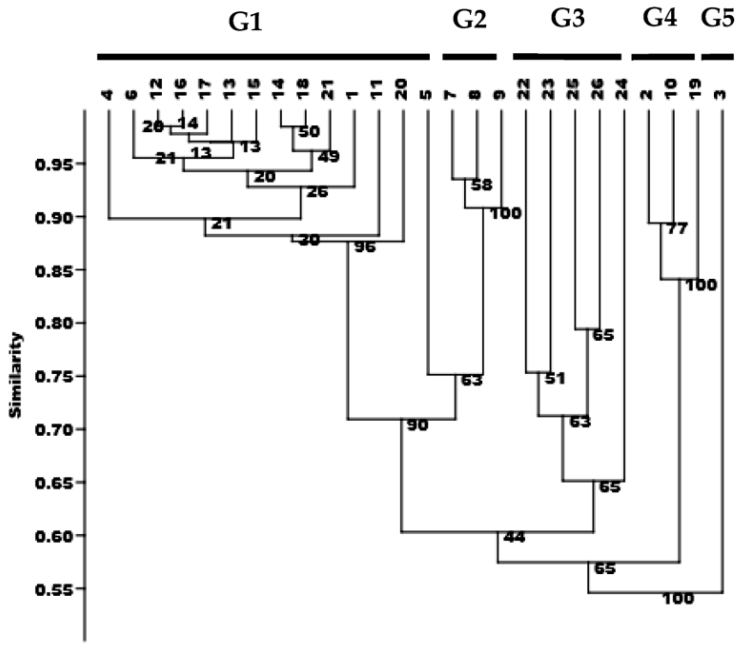

Figure 6. Dendrogram of 26 kumquats generated by Jaccard coefficient and UPGMA clustering method based on SRAP molecular markers. Note: The numbers in front of the branches are bootstrap values. The genotype codes: 1. NB Jindan; 2. DG Jindou; 3. JG Zazhong; 4. WZ Luofu; 5. NB Luofu; 6. WZ Jindan; 7. Sijiju; 8. Wenzhouju; 9. Shouxingju; 10. Dajindou; 11. NB Luowen; 12. RA Jingan; 13. FY Jingan; 14. CM Jingan; 15. G Jingan1; 16. G Jingan2; 17. YS Jingan; 18. 15-1; 19. Shanjingan; 20. LY Jingan; 21. HP Jingan; 22. FC-1; 23. FC-2; 24. FC-3; 25. FC-4; 26. FC-5.

Table 6. The unique kumquat genotype-specific markers.

Code	Genotypes	Unique Specific Markers
1	NB jindan	M1E15(1800−), M3E17(250−)
2	Daguojindou	M1E23(400−)
3	Jinganzazhong	M1E22(250+), M4E17(500+), M9E23(700−), M10E13(200+)
4	WZ luofu	None
5	NB luofu	M2E21(400−), M3E17(450+), M11E21(250+)
6	WZ jingdan	M2E21(450+), M16E9(350−)
7	Sijiju	M4E12(1300−)
8	Wenzhouju	M17E2(530−)
9	Shouxingju	None
10	Dajindou	M3E17(100+), M4E17(250−)
11	NB luowen	None
12	RA jingan	None
13	FY jingan	None
14	CM jingan	None
15	Guijingan1	None
16	Guijingan2	None
17	YS jingan	None
18	F15-1	None
19	Shanjingan	M1E22(300−)
20	LY jingan	M7E4(500+)
21	HP jingan	None
22	FC-1	M2E17(500+), M2E17(1000+)
23	FC-2	M4E7(500−)
24	FC-3	None
25	FC-4	M4E17(300+)
26	FC-5	M14E12(1300+)

Note: Special marker is denoted in SRAP primer pair code plus marker base pair in parentheses, + means the presence of the band, − absence of the band.

By using the unique specific SRAP markers, the 14 kumquat genotypes could be clearly distinguished. For the remaining 12 accessions without unique specific markers, they needed marker combinations to identify them. FC-3 did not have a unique specific marker; however, it shared the specific marker M3E17(180+) with FC-1, FC-4, and FC-5, and the latter three accessions had unique specific markers for each. Obviously, FC-3 could be distinguished from them by the combination of M3E17(180+) with the unique specific markers of FC-1, FC-4, and FC-5. Shouxingju is a hybrid kumquat without a unique specific marker, but it shared the specific marker M18E22(300+) with Wenzhouju, Sijiju, and Jinganzazhong. The combination of M18E22(300+) with the unique specific markers of Wenzhouju, Sijiju, and Jinganzazhong made Shouxingju distinguishable (Table 7).

Table 7. The combinations of specific markers for kumquat identification.

Genotypes	Common Specific Markers	Combination of Unique Markers
FC-3	M3E17(180+) FC-1; FC-4; FC-5	FC-1 M2E7(500+); FC-4 M4E17(300+); FC-5 M14E12(1300+)
Shouxingju	M18E22(300+) Wenzhouju;Sijiju; Jinganzazhong	Wenzhouju M17E2(530−); Sijiju M4E12(1300−); JinganzazhongM1E22(250+)
NB luowen	M10E7(850−) WZ Luofu; NB luofu; Wenzhouju	WZ Luofu M10E7(320−); NB luofu M2E21(400−), M3E17(450+), M11E21(250+); Wenzhouju M17E2(530−)
WZ Luofu	M10E7(320−) NB luofu; Wenzhouju; Shanjingan	NB luofu M2E21(400−), M3E17(450+), M11E21(250+); Wenzhouju M17E2(530−); Shanjingan M1E22(300−)

Note: Special marker is denoted in SRAP primer pair code plus marker base pair in parentheses, + means the presence of the band, − absence of the band.

There existed another pair of genotypes, WZ luofu and NB luowen that originated in Zhejiang, that remained indistinguishable. NB luowen and WZ Luofu shared the marker M10E7(850−) with NB luofu and Wenzhouju. Nevertheless, NB luofu possessed three unique markers [M2E21(400−), M3E17(450+), and M11E21(250+)], and Wenzhouju had the M17E2(530−) unique marker, which allowed them to be discriminated from NB luowen and WZ Luofu. In addition, WZ Luofu shared the specific marker M10E7(320−) with NB luofu, Wenzhouju, and Shanjingan but not with NB luowen, allowing discrimination of the two genotypes. WZ Luofu was easily separated from other marker-sharing genotypes due to their unique markers [NB luofu M2E21(400−), M3E17(450+), and M11E21(250+); Wenzhouju M17E2(530−); and Shanjingan M1E22(300−)] (Table 7).

There were still eight accessions that could not be distinguished. These were cultivars and their bud mutants cultivated mainly in Guangxi, including RA jingan, HP jingan, CM jingan, FY jingan, F15-1, YS jingan, Guijingan1, and Guijingan2 (named Guangxi cultivar group) (Table 6). After a careful search through all the SRAP markers, Guangxi cultivar group was found to possess two group-specific markers [M1E23(800−) and M7E4(1050+)] with NB jindan, WZ jingdan, LY jingan, WZ luofu, and NB luofu (Table 8). As the latter five genotypes could be distinguished from the Guangxi cultivar group by their unique specific markers, the members of the Guangxi cultivar formed a special group separated from all the other kumquat accessions (Table 8).

Successively, genotype-specific markers within the Guangxi cultivar group were checked. Guijingan1, HP jingan, and FY jingan had a genotype-specific marker for each [M1E22(740−), M10E7(710−), and M3E17(90−), respectively], permitting easy discrimination from other group members. For others, bi- or tri-markers were detected. CM jingan and HP jingan had a bi-specific marker M2E21(480−), making CM jingan distinguishable from HP jingan by its specific marker M10E7(710−). YS jingan and F15-1 shared another bi-specific marker M14E12(760−), while Guijingan2, YS jingan, and HP jingan possessed a tri-specific marker M14E12(700−). By the two combinations of markers, HP jingan was first discriminated by its single marker, YS jingan occupied both markers thus distinguishable, and thereafter F15-1 only presented M14E12(760−) and Guijingan2 solely M14E12(700−).

The last member in the group was RA jingan, which did not have any specific marker to distinguish it from the other seven members (Table 9).

Table 8. Guangxi cultivars' group-specific markers for identification.

Group Markers	Shared Genotypes with	Unique Markers for Discrimination
M1E23(800−)	NB jindan, WZ luofu, WZ jingdan LY jingan	NB jindan M1E15(1800−), M3E17(250−) WZ luofu M10E7(320−) WZ jingdan M2E21(450+), M16E9(350−) LY jingan M7E4(500+)
M7E4(1050+)	NB jindan NB luofu WZ jingdan LY jingan	NB jindan M1E15(1800−), M3E17(250−); NB luofu M2E21(400−), M3E17(450+), M11E21(250+) WZ jingdan M2E21(450+), M16E9(350−) LY jingan M7E4(500+)

Note: Special marker is denoted in SRAP primer pair code plus marker base pair in parentheses, + means the presence of the band, − absence of the band.

Table 9. Genotype identification within Guangxi cultivars group.

Marker Types	Genotypes	Markers for Discrimination
Single marker	Guijingan1 HP jingan FY jingan	M1E22(740−) M10E7(710−) M3E17(90−)
Bi/Tri- markers	CM jingan + HP jingan YS jingan + F15-1 Guijingan2 + YS jingan + HP jingan	M2E21(480−) M14E12(760−) M14E12(700−)
No specific marker	RA jingan	

Note: Special marker is denoted in SRAP primer pair code plus marker base pair in parentheses, + means the presence of the band, − absence of the band.

4. Discussion

The genetic diversity of plant species is the basis of their survival and evolution, and genetic research is an effective method to evaluate and quantify genetic variation [30]. With the development of DNA fingerprinting technology, molecular markers have been widely used in molecular taxonomy, variety identification, and marker-assisted selection in different plants [31,32]. SRAP is a PCR-based technique that has been widely used in plant germplasm diversity, variety identification, genetic mapping, and gene cloning in recent years [33] in various crops, including coffee [29], kiwifruit [34], and litchi [35]. In this study, 19 combinations of SRAP primers were used to determine the genetic diversity of 26 kumquat accessions. Out of the 104 bands amplified, 90 (86.54%) were polymorphic, which made it possible to analyze the genetic diversity and to identify all 26 kumquat accessions. These results indicate that SRAP markers are useful for kumquat genetic diversity analysis and genotype identification.

In the studies on germplasm diversity, principal coordinate analysis, structural analysis, and UPGMA cluster analysis are often utilized to carry out data analysis [16,29]. In the present work, the SRAP data of 26 kumquat genotypes were analyzed by using these three methods. In the principal coordinate analysis, though PC1 and PC2 only contained 54.05% of all the information, PC1 analysis results played an important role in the classification of wild and cultivated kumquats, which were further subdivided into five subgroups by PC2. The results of structural analysis showed that 26 kumquat accessions were first divided into two groups, wild kumquats and cultivated, and then into five groups. However, NB luofu could not be classified into any of the groups as it had genetic background components of four groups. This might suggest that NB luofu could be of hybrid origin. UPGMA cluster analysis showed that 26 kumquat genotypes were also divided into 5 groups. Surprisingly, all 26 kumquat accessions were classified into 5 identical groups with the 3 data processing

methods, which might indicate that the SRAP markers were stable and reliable. Here, the results might reflect the genetic differences between the kumquat accessions.

Other than genetic origin, geographic regions also affect kumquat biodiversity. Among the 26 tested kumquat accessions, 14 were from Guangxi and 12 from other provinces. As mentioned above, genetic background classified the 14 Guangxi kumquat germplasm resources into 3 groups according to their origins: wild kumquat, hybrid kumquat, and cultivated varieties. The results indicate the rich genetic diversity of the Guangxi kumquat germplasm. However, in comparison with the kumquat germplasm from other provinces, the Guangxi kumquat germplasm resulted in different groups, even belonging to the same cultivated or wild types. The Guangxi wild kumquat (FC-1, FC-2, FC-3, FC-4, and FC-5) remained in different groups from the wild ones from Hunan (Shanjingan, Dajindou and Dagujindou). The Hunan wild kumquats belong to *F. hindisii*, whose trees are dwarf with small leaves, have fruits like a bean in size, and have twigs with long thorns. On the other hand, the Guangxi wild kumquat genotypes have big trees over 5 m tall, small leaves the same as those of *F. hindisii* in size, and fruit much bigger than that of *F. hindisii* and a little smaller than that of *F. crassifolia*. The differences in morphological characteristics and SRAP profiles indicate that the Guangxi wild kumquat might be a new *Fortunella* species; obviously, such a suggestion needs further investigation for confirmation.

Jinganzazhong, a Guangxi kumquat hybrid, was collected from Gupu Mountain in Hezhou, where it was remote and was hardly introduced to anything from outside the area. In fact, its SRAP profiles were distinct from those of the well-known intergenus hybrids (Shouxingju, Sijiju, and Wenzhouju). They might have different parentage in origin, and successive identification is necessary.

Though the cultivated kumquat varieties usually formed in one group in the classification, indicating a close genetic relationship, eight cultivars from Guangxi and six from Hunan and Zhejiang were clustered into two subgroups. This suggests that the cultivated kumquat in Guangxi also had certain genetic diversity and specificity compared with those from other provinces. Thorough studies are necessary to ascertain whether this differentiation is due to their genetic origin or geographic evolution effects.

Zhu et al. suggested that the genus *Fortunella* consisted of two populations: cultivated kumquat and Hongkang (wild) kumquat [16]. The results of this study revealed that the kumquat germplasm was divided into wild and cultivated kumquat groups in Principal Coordinate Analysis (PC1) and Population Structure Analysis (at K = 2). Hereby, it seems that *Fortunella* may be roughly divided into wild and cultivated genotypes.

Some researchers intended to put kumquat into *Citrus* (*Citrus japonica* Thunb) [12]. Wang et al. supported the incorporation of kumquat into *Citrus*, but the traditional kumquat should have three species: *F. venosa*, *F. hindsii*, and *F. japonica* [15]. In the present study, the wild kumquat genotypes were divided into the Guangxi wild kumquat group and the golden bean group. All the accessions of *F. hindsii* had a very close relationship and did not appear to be able to divide into two species. The present study did not provide sufficient data to form opinions on the suggestion of putting kumquat into *Citrus*.

The cultivated kumquat includes three species: Luowen (*F. japonica*), Luofu (*F. margarita*), and Jindan (*F. crassifolia*) [2]. Zhu et al. found that there was a clear genetic structure of "*F. margarita–F. crassifolia*" in cultivated kumquats. The Luowen may have originated from a cross or backcross between Luofu and Jindan, but all three cultivated species deserved the status of "species" [16]. After the analyses by RAPD and CAPs of chloroplasts, Yasuda et al. suggested that the three cultivated kumquat species should be combined into one species (*F. margarita* complex) [14]. Here, we found that the cultivars derived from Luowen, Luofu, and Jindan could not be clearly divided into three species, and it is possible that the cultivated kumquat might not be able to be divided into the three species Luowen, Luofu, and Jindan, as there is insufficient genetic information.

Genotype-specific markers are an efficient tool for identifying germplasm resources. SRAP markers have been successfully applied to the variety identification of fruit trees such as apple [36], kiwifruit [37], and grape [38]. The kumquat is a perennial woody

plant with a complex genetic background. Most of the cultivated varieties have originated from bud mutation with a narrow genetic background, which usually leads to difficulty in distinguishing by molecular markers. Therefore, the present results, with SRAP markers allowing identification of all the tested kumquat accessions, have a certain importance for kumquat genetic research. In this study, unique genotype-specific SRAP markers were detected for 14 kumquat genotypes, which made it possible to positively identify them. For the remaining 12 accessions without genotype-specific markers, they (including the cultivated varieties that originated from bud mutation) were distinguished by various combinations of markers. These specific markers will be useful for kumquat cultivar discrimination, nursery identity verification, and hybrid identification, which will be valuable in kumquat breeding and cultivar patent protection.

Wild kumquats in Guangxi have been in a wild state for a long time and exposed to different environmental stresses such as drought, high/low temperatures, and various pests and diseases. The kumquat germplasm has demonstrated strong adaptability to adversity and possesses tolerance to abiotic and/or biotic stresses. It may be used in genetic improvement for increasing the tolerance to stresses. What is more, this wild kumquat is mono-embryotic and of short juvenility, whose characteristics permit it to be used as a female parent with high efficiency in cross-breeding.

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