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Development and Application of Polymorphic SSR Markers in *Luffa cylindrica*

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Abstract

Two published genomes of *Luffa cylindrica*, 'SG2019' and 'SO3', were used to develop polymorphic SSR markers for *L. cylindrica* by searching and comparing the SSR motifs at the same site in the two genomes. Based on the SSR search conditions and primer design criteria, 2130 polymorphic SSR markers for *L. cylindrica* were developed. The main motif type was dinucleotide, accounting for 80.28% of the total; the main motif units were AT/AT and AAT/ATT, accounting for 87.80% of the total. Furthermore, 40 polymorphic SSR markers developed in this study were randomly selected and amplified in 24 *Luffa* samples. According to the results, the 40 polymorphic SSR markers showed an amplification rate of 100% and a polymorphism rate of 80%. Cluster analysis classified the 24 *Luffa* samples into two main groups, *L. cylindrica* and *L. acutangula*. Overall, the polymorphic SSR markers developed in this study display a high polymorphic rate and reliable utilization value.

Additional key words: genetic diversity, genomic prediction, polymorphic information content, sponge gourd, SSR motif repeat, validation of polymorphism

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Introduction

The genus *Luffa* comprises two cultivated species, *Luffa cylindrica* (sponge gourd) and *L. acutangula* (ridged gourd). *L. cylindrica* is a common vegetable crop mostly cultivated in India, China, and Southeast Asia (Filipowicz et al., 2014). It has high nutritional value and a delicious taste. Specifically, *L. cylindrica* is rich in carbohydrates and various mineral elements. It also contains various biologically active substances such as glycosides, alkaloids, and flavonoids (Yan et al., 2011). *L. cylindrica* is also a medicinal plant with special medicinal value, including antimicrobial and anti-cancer properties (Sharma et al., 2019). Compared with other cucurbit crops, research on the genetics and breeding of *L. cylindrica* has lagged. Until now, most of the new varieties have been improved by traditional methods (Dai et al., 2016).

High-throughput sequencing has accelerated large-scale development of crop molecular markers. However, screening polymorphic molecular markers is still an expensive approach. Simple sequence repeats (SSRs), also known as microsatellite markers, have high stability and polymorphism and thus have become one of the most applied mainstream molecular markers (Ellegren, 2004; Wang et al., 2015).

Researchers have mainly developed SSR markers of *L. cylindrica* through transcriptome sequencing prior to whole-genome sequencing of the species (Wu et al., 2014; Zhu et al., 2016b). With the completion of whole-genome sequencing of *L. cylindrica* (Wu et al., 2020; Zhang et al., 2020; Pootakham et al., 2021), 284 966 SSR markers in the whole genome were developed (Qiao et al., 2021). However, large-scale development of polymorphic SSR markers in *L. cylindrica* by genome prediction remains poorly explored.

In this study, two genomic sequences of *L. cylindrica* 'SG2019'(Zhang et al., 2020) and 'SO3' (Pootakham et al., 2021), were utilized to develop polymorphic SSR markers for *L. cylindrica*. SSR loci were identified at the same site with different repeats between the two genomes. Furthermore, 40 pairs of predicted polymorphic SSR primers were randomly selected for SSR genotyping of 24 *Luffa* samples, and their high polymorphic rate was verified. The polymorphic SSR markers developed in this study can improve the efficiency of SSR markers and accelerate the process of marker-assisted selection in *L. cylindrica*.

Materials and Methods

L. cylindrica Genome Source

Polymorphic SSR markers were developed based on two published genomes of *L. cylindrica*. The two genomes were 'SG2019' (Zhang et al., 2020) from cngbdb (https://db.cngb.org/), accession number cnp0000780, and 'SO3' (Pootakham et al., 2021) from NCBI (https://www.ncbi.nlm.nih.gov/), accession number GCA_ 012295205.1.

Plant Materials

A total of 24 *Luffa* samples (Table 1) were used to verify the polymorphism of developed SSR markers. The 24 *Luffa* samples included landraces and commercial cultivars of *L. cylindrica*, except for 'Shengyou', 'Meizhou' and 'Shengyou × Pingguo F_1' (first generation of interspecific hybrid between *L. acutangula* 'Shengyou' and *L. cylindrica* 'Pingguo').

Development of Polymorphic SSR Markers

MISA (http://pgrc.ipk-gatersleben.de/misa/) was used to search for SSR loci of the two *L. cylindrica* genomes 'SG2019' and 'SO3'. The search parameters were: (1) the SSR length was larger than 22 bp; (2) the length of sequences on both sides of the SSR site was set to 150 bp; (3) motif repeat times ≥ 4; (4) The length of the motif was 2 − 6, and the corresponding SSR motif types were dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, and hexanucleotide.

Primer3 software was used to design primers for SSR loci found in the two genomes based on the following parameters: 'prime_product_size_range = 80-160 80-240 80-300; prime_opt_size = 24; prime_min_size = 20; prime_max_size = 28; prime_opt_tm = 63; prime_min_tm = 60; prime_max_tm = 65'. After primer design, the upstream and downstream primer sequences of each SSR locus were aligned back to the reference genome of 'SG2019', and only the SSR loci specifically aligned to the primer sequences were retained.

Finally, using 'SG2019' as the reference genome, SSR loci with different motif repeats between 'SG2019' and 'SO3' were identified using the Mummer program (http://mummer.sourceforge.net/manual/).

Table 1. Basic information of 24 *Luffa* samples

No.	Germplasm	Type	Origin	Main fruit traits
1	Shengyou	*L. acutangula*, Commercial cultivar	Guangzhou, Guangdong Province, China	Long stick-shaped, ridged, smooth skin
2	Pingguo	*L. cylindrica*, Commercial cultivar	Shouguang, Shandong Province, China	Round-shaped, non-ridged, smooth skin
3	Shengyou × Pingguo F_1	Interspecific hybrid F_1	Hybrid F_1 from Shengyou and Pingguo, China	Long stick-shaped, ridged, smooth skin
4	Shuangfeng No.19	*L. cylindrica*, Commercial cultivar	Shantou, Guangdong Province, China	Long stick-shaped, non-ridged, smooth skin
5	Shuangfeng No.20	*L. cylindrica*, Commercial cultivar	Shantou, Guangdong Province, China	Long stick-shaped, non-ridged, smooth skin
6	Shuangfeng No.21	*L. cylindrica*, Commercial cultivar	Shantou, Guangdong Province, China	Long stick-shaped, non-ridged, smooth skin
7	Zaoxiang No.1	*L. cylindrica*, Commercial cultivar	Hengyang, Hunan Province, China	Long stick-shaped, non-ridged, smooth skin
8	Zaozhou No.2	*L. cylindrica*, Commercial cultivar	Hengyang, Hunan Province, China	Long stick-shaped, non-ridged, wrinkled skin
9	Duobaorou	*L. cylindrica*, Commercial cultivar	Shantou, Guangdong Province, China	Long stick-shaped, non-ridged, smooth skin
10	Lvyou No.1	*L. cylindrica*, Commercial cultivar	Nanjing, Jiangsu Province, China	Long stick-shaped, non-ridged, smooth skin
11	Qingxiang	*L. cylindrica*, Commercial cultivar	Nanjing, Jiangsu Province, China	Long stick-shaped, non-ridged, smooth skin
12	Baixueyu	*L. cylindrica*, Landrace	Jinhua, Zhejiang Province, China	Long stick-shaped, non-ridged, smooth skin
13	Liulin 1	*L. cylindrica*, Landrace	Bazhong, Sichuan Province, China	Long stick-shaped, non-ridged, smooth skin
14	Baiyu No.1	*L. cylindrica*, Commercial cultivar	Changsha, Hunan Province, China	Long stick-shaped, non-ridged, smooth skin
15	Shengyou 20	*L. cylindrica*, Commercial cultivar	Nanjing, Jiangsu Province, China	Long stick-shaped, non-ridged, smooth skin
16	Changlv	*L. cylindrica*, Commercial cultivar	Beijing, China	Long stick-shaped, non-ridged, smooth skin
17	Liulin 2	*L. cylindrica*, Landrace	Bazhong, Sichuan Province, China	Long stick-shaped, non-ridged, smooth skin
18	Juxing No.1	*L. cylindrica*, Commercial cultivar	Yantai, Shandong Province, China	Long stick-shaped, non-ridged, smooth skin
19	Yunyong	*L. cylindrica*, Landrace	Foshan, Guangdong Province, China	Long stick-shaped, non-ridged, smooth skin
20	Fengyou No.3	*L. cylindrica*, Commercial cultivar	Shantou, Guangdong Province, China	Long stick-shaped, non-ridged, smooth skin
21	Xiangyubai	*L. cylindrica*, Commercial cultivar	Zhuzhou, Hunan Province, China	Long stick-shaped, non-ridged, smooth skin
22	Meizhou	*L. acutangula*, Landrace	Meizhou, Guangdong Province, China	Long stick-shaped, ridged, smooth skin
23	Changfeng	*L. cylindrica*, Commercial cultivar	Liaoyang, Liaoning Province, China	Long stick-shaped, non-ridged, smooth skin
24	Yulin	*L. cylindrica*, Landrace	Yulin, Guangxi Province, China	Long stick-shaped, non-ridged, smooth skin

SSR Genotyping and Polymorphic Analysis

The SSR reaction system and amplification were performed as described in a previous report (Cui et al., 2017). The amplification products were resolved in 6% polyacrylamide gels and stained with silver. When an SSR marker gave an amplification product on at least one of the samples, this indicated that the marker could be amplified. Based on the electrophoresis results, a clear band was marked as '1' and an absence of a band at the same position was marked as '0', forming a '0-1' matrix. The data were converted into different formats using DataFormater software (Fan et al., 2016) as required. The polymorphism information content (PIC) of each SSR marker was calculated using Powermarker software (Liu and Muse, 2005). The cluster analysis was analyzed using NTSYS-pc software (http://www.appliedbiostat.com/ntsyspc/ntsyspc.html), whereas the population structure was analyzed using structure software (Pritchard et al., 2000).

Results

Characterization of Polymorphic SSRs in *L. cylindrica*

A total of 21249 and 21303 SSR loci were identified in *L. cylindrica* genomes 'SG2019' and 'SO3', respectively. Then, taking the 'SG2019' genome as a reference, 4613 (21.71%) concomitant SSR loci and 2130 (10.02%) SSR loci with different motif repeats were found by comparing the SSR motif repeats in the two *L. cylindrica* genomes (Table 2, and Suppl. Table 1). Among the 2130 predicted polymorphic SSR loci, dinucleotides accounted for the largest proportion (1710; 80.25%), followed by trinucleotides (322; 15.12%). The number of tetranucleotides, pentanucleotides, and hexanucleotides was 40 (1.88%), 19 (0.89%), and 39 (1.83%), respectively. In addition, the 2130 polymorphic SSR loci contained 54 types of motif units, of which AT/AT and AAT/ATT accounted for 87.80% of the total number.

Table 2. Polymorphic SSR motifs predicted from two *L. cylindrica* genomes

Motif type	Motif unit	Number	Proportion (%)
Dinucleotide	AT/AT	1 604	75.31
	AG/CT	97	4.55
	AC/GT	9	0.42
Trinucleotide	AAT/ATT	266	12.49
	AAG/CTT	48	2.25
	ATC/ATG	4	0.19
	ACC/GGT	2	0.09
	AGG/CCT	2	0.09
Tetranucleotide	AAAT/ATTT	23	1.08
	AAAG/CTTT	9	0.42
	AATT/AATT	3	0.14
	ACAT/ATGT	3	0.14
	Other	2	0.09
Pentanucleotide	AAAAG/CTTTT	3	0.14
	AAAAT/ATTTT	3	0.14
	ACCCC/GGGGT	2	0.09
	AATAC/ATTGT	2	0.09
	Other	9	0.42
Hexanucleotide	AAAAAG/CTTTTT	7	0.33
	AAAAAC/GTTTTT	4	0.19
	AACAAG/CTTGTT	2	0.09
	AAGATG/ATCTTC	2	0.09
	AATTCC/AATTGG	2	0.09
	Other	22	1.03
Total		2 130	100

Validation of Polymorphic SSR Markers in *Luffa* Samples

To validate the polymorphism of the newly developed polymorphic SSR markers, we randomly selected 40 SSR markers that were distributed on all chromosomes and contained different motif types to genotype the 24 *Luffa* samples. Amplification results showed that all 40 SSR markers bound to specific regions in some or all the 24 *Luffa* samples, with a 100% amplification rate. Furthermore, 32 out of the 40 SSR markers showed polymorphism in the 24 *Luffa* samples, with an 80% polymorphic rate. The 32 SSR markers amplified 2−5 alleles in the 24 *Luffa* samples, with an average of 3.16 (Table 3). The PIC values of the 32 SSR markers in the 24 *Luffa* samples ranged from 0.08 to 0.65, among which the PIC values of pLC0777, pLC0767, and pLC1223 were 0.65, 0.57, and 0.50, respectively, indicating a high level of polymorphism (Fig. 1).

Table 3. Sequence information and polymorphism verification of 32 polymorphic SSR primers in *Luffa*

Marker name	Chromosome	SSR motif	Motif repeat in SG2019	Motif repeat in SO3	Primer sequence (5′-3′)	Allele number	PIC
pLC0033	Chr 1	AT	17	15	TCCACTAAGGAAAATTCAAATGGG TCTGAAATAACAGGGATTGTGACAG	2	0.20
pLC0144	Chr 1	CAATC	6	5	TCTTCTTCTTCCAATCCAATCCAA GAAAGGAGAGGGTGAAGGACAGAG	3	0.33
pLC0145	Chr 1	GGCTT	8	10	GGATCTTTTCTTTTTGGCTCCTCA CCAATTCCAAACCAAGCATTCTTAT	3	0.17
pLC0288	Chr 2	TAA	14	8	AAATTTCCGATCCCTCTGTTTTG GCAATCTTCGCTAGCTTGATTCAT	3	0.34
pLC0305	Chr 2	TCTTTC	5	4	AGTAAACCCCATTTCAGCCCTTTA GGGGGCTATAAGGAGGAAAACAAT	3	0.41
pLC0307	Chr 2	TGGTGA	7	4	GTTTCGACGATGGCAACAACT TGCTTTCCTTTATTCTTTCTCTCTTCTT	5	0.46
pLC0426	Chr 3	AG	11	12	AAATTGGAGAGAAGTTTCTTGGGC TCCGATATCCCTTTCCATTTTTCT	2	0.17
pLC0455	Chr 3	GGAGAT	5	4	TTGAAATAACTTATCGCGTTTCGG AAAATCTATTCTTCTCCCGCGTTC	3	0.36
pLC0612	Chr 4	AAG	13	12	TCACCAATCTATCACCAATCTATCAA TCTGTTCAATTCTAACTTTCCCCC	4	0.33
pLC0621	Chr 4	AAAAT	6	5	GCTGCTTTTAACAAGGGAAATCCT ACAATCACGTTTTTGCCATTTTTC	2	0.17
pLC0767	Chr 5	TAT	14	12	AAAAGTTTCTTCCACATTCATACTTTTT CATCGAGTATCGACTCCACATCTA	5	0.57
pLC0769	Chr 5	TGTA	11	9	CTTTGCATCCCCTACATTTTGAAC CCAAACAAGCTTTAGCTTTAAATTTGC	3	0.29
pLC0774	Chr 5	AGAGAA	6	5	CGGACCTTTCAACCAACAAAAATA CCTCACGTTCCTTCATATCAGATTTC	4	0.45
pLC0777	Chr 6	TTCTTG	8	5	GTTCGTTGTTTCTTGAACCAGTCC ATTCTTGTCCACAACCAAACCCTA	4	0.65
pLC0943	Chr 6	AAAT	6	8	TCGAAATCACATTTTCACATTCTCA CCAGAAGTTCAAATCTCTCACCTCA	3	0.31
pLC1062	Chr 7	AT	17	16	CACTCAACACTGCATAGTTTTCGG CCAAACAACATCTACAAATTATGGCTC	4	0.27
pLC1092	Chr 7	ATA	22	23	AATTCACATCTTGACCTCAATCCC ATAGCACATCTTCAACTCCATCCC	2	0.08

Table 3. Sequence information and polymorphism verification of 32 polymorphic SSR primers in *Luffa* (Continued)

Marker name	Chromosome	SSR motif	Motif repeat in SG2019	Motif repeat in SO3	Primer sequence (5′-3′)	Allele number	PIC
pLC1099	Chr 7	TGGGG	6	5	AAAATTGGATCTGCTTACCTTTGTTAAA AAATTTTCCCCACAAGAATCAGC	2	0.17
pLC1101	Chr 7	GTATCG	5	6	TCATTTCCCAAATTTCTGATCTTTTT ACATTCAAAACAAACCCACCAATC	3	0.08
pLC1223	Chr 8	TCTTGT	6	7	TATGGGATCTTTGCTGCAGGATTA TCCCCTTTAGGATCCATTTCTTGT	4	0.50
pLC1362	Chr 9	TTA	10	8	CGTCAACATCTTTGTTGGCAATAG TGCTTTACGTTCTCCAAATTCCAT	3	0.34
pLC1367	Chr 9	TTCA	8	7	TGAGTGATGTGTGAAAAAGAAGTGC GGCCAAAGTGTATGAGCGTAAAAC	3	0.29
pLC1369	Chr 9	ACAAT	10	9	AAAACGCTTTCAAAAGCAAAACCT GGTCCAAATTTTATCCATTAGCCC	5	0.29
pLC1374	Chr 9	GGAACA	5	7	AAACCGAGAGGAAACGGAGAGTTT GGGGTACAGATCCTGTGAGTCTGA	4	0.19
pLC1469	Chr 10	TTAA	7	6	TCTCCCTTCCTCTTCTCTCCTCTT GTGGGAGGGAATATGTGACTTACG	2	0.08
pLC1568	Chr 11	AT	18	15	AAATGTTCATGATCACCTTTTGGG ACATAATGTTGACAAGCAAAGCCA	3	0.29
pLC1575	Chr 11	TTA	10	11	GCCTTCATCATTCTGATGTGGATT TTTGTACTGGGCAGCATGTAACAG	2	0.17
pLC1588	Chr 11	ATTGGA	6	7	TGTTGGAGATTGGAGAGTGATGAG TGTTTGGTTTCCCAGAAATATGCT	3	0.33
pLC1881	Chr 12	TTTA	7	6	CATGCTTTTAATAAACCAGAGTTCCC GGTAATCTCTACCCCTTGCCCTTA	3	0.46
pLC1886	Chr 12	TATAGA	6	5	CAAAAAGTTTCTCCTCATTGTGGC TTCGGTCAAAGAAGAGACAAAAGTG	3	0.39
pLC2117	Chr 13	TTCT	10	11	CTTTGTTTGTGCGACAATCGG CGTGCAAAACCCAAATCCATA	3	0.41
pLC2122	Chr 13	AATA	8	6	AATTGTCTCTCCGGTGATCTGAAA TTTTGTTGCAGAGTGATTCCAAAA	3	0.24
Average						3.16	0.31

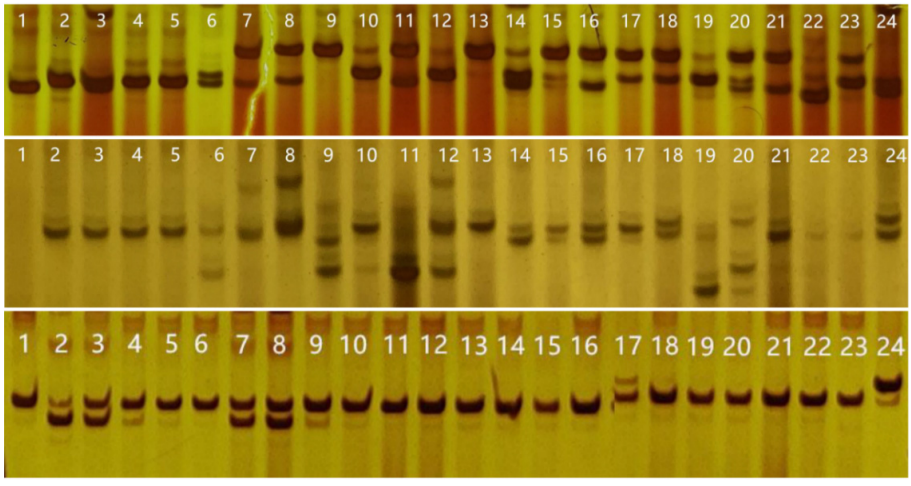

Fig. 1. Amplification results of three pairs of highly informative *Luffa* SSR primers. From top to bottom, three pairs of *Luffa* SSR primers are pLC0777, pLC0767, and pLC1223; 1 to 24 indicate the numbers of 24 *Luffa* samples.

Application of Polymorphic SSR Markers in Genetic Diversity Analysis

Cluster analysis was conducted on the 24 *Luffa* samples using 32 polymorphic SSR markers from *L. cylindrica*. The 24 *Luffa* samples were divided into two groups at a genetic similarity coefficient of 0.47 (Fig. 2).

Group Ⅰ included two *L. acutangula* samples, 'Shengyou' (No. 1) and 'Meizhou' (No. 22), whereas group II included 22 *Luffa* samples. At a genetic similarity coefficient of 0.80, the 22 *Luffa* samples in group II were divided into four subgroups: group II-1 included two samples, 'Pingguo' (No. 2) and 'Shengyou × Pingguo F_1' (No. 3); group II-2 included one *L. cylindrica* sample, 'Zaozhou No.2' (No. 8); group II-3 included four *L. cylindrica* samples, 'Yulin' (No. 24), 'Fengyou No. 3' (No. 20), 'Changfeng' (No. 23), and 'Juxing No.1' (No. 18); and group II-4 included the remaining 15 *L. cylindrica* samples.

The population structure of the 24 *Luffa* samples was analyzed using structure software. The structure analysis results were consistent with the clustering results and specifically reflected the kinship of different samples (Fig. 3). For example, a population structure of K ranging from 2 to 4 indicated that the interspecific hybridization, 'Shengyou × Pingguo F_1' (No. 3), came from two parents. The commercial cultivar *L. cylindrica* 'Changfeng' (No. 23) was more likely to contain a small fraction of genetic background from *L. acutangula* germplasm.

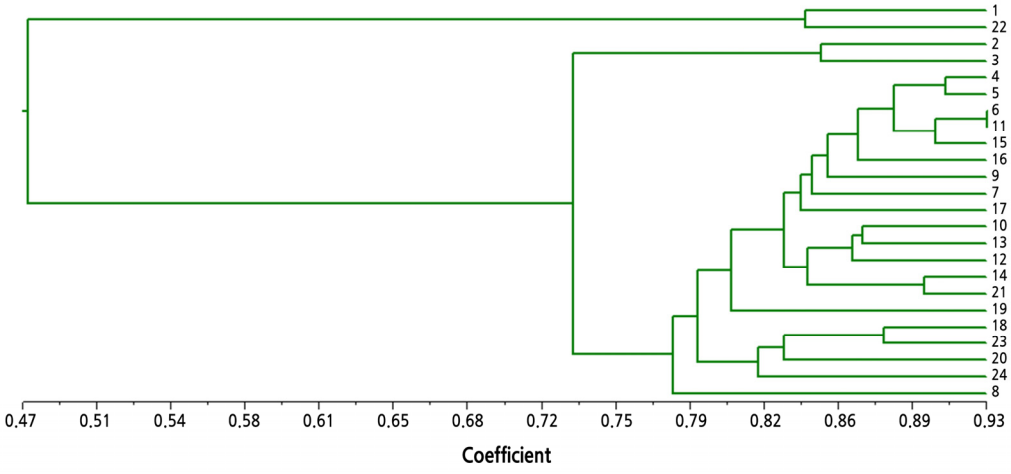

Fig. 2. Cluster analysis of 24 *Luffa* samples.

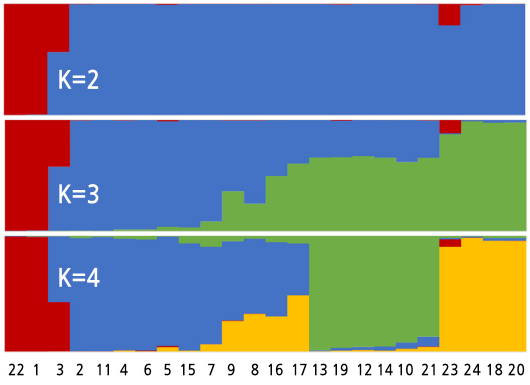

Fig. 3. Population structures of 24 *Luffa* samples. K = 2-4 represents the number of population structures in 24 *Luffa* samples.

Discussion

Genome-wide SSR markers of *L. cylindrica* have recently been developed (Qiao et al., 2021). The availability of efficient and reliable polymorphic markers significantly reduces the screening costs and delays during the validation of large-scale SSR markers. There are few reports on the application of SSR markers in *L. cylindrica.* For example, there are a few reports on the application of SSR markers for the analysis of genetic diversity of germplasm resources (Liu et al., 2010), the construction of genetic maps and mapping of flowering time (Wu et al., 2016), and the identification of hybrid purity (Zhu et al., 2020).

In this study, 2130 SSR markers showing polymorphism between *L. cylindrica* genomes 'SG2019' and 'SO3' were obtained by comparing their genomic sequences. The number of dinucleotide and trinucleotide SSR loci in the genome of *L. cylindrica* was predicted to be 54363 and 28972, respectively (Qiao et al., 2021). However, the proportion of dinucleotide SSR loci was 80.28%, far exceeding the proportion of trinucleotide SSR loci (15.12%), indicating that dinucleotide SSR loci are the main type of polymorphism in the *L. cylindrica* genome. The 2130 polymorphic SSR markers contained 54 kinds of motif units, which was much lower than the 211 kinds in the whole genome of *L. cylindrica* (Qiao et al., 2021), indicating that the polymorphic SSR markers of *L. cylindrica* are skewed towards some types of motif units. Among the polymorphic SSR markers of *L. cylindrica*, AT/AT and AAT/ATT had the largest number of motif units, consistent with the *L. cylindrica* genome. In this study, the designed SSR primer sequences were aligned back to the reference genome, and those that aligned uniquely were retained to ensure the amplification of the SSR markers. A total of 40 SSR markers were amplified in 24 *Luffa* samples, with an amplification rate of 100%. Among the 40 SSR markers verified, 32 were polymorphic with a polymorphic rate of 80%, which was higher than that reported previously (Wu et al., 2014; Zhu et al., 2016b; Qiao et al., 2021).

Based on the standard of polymorphism level (Botstein et al., 1980), only three out of the 32 polymorphic SSR markers verified in this study were highly informative (PIC $\geq$ 0.50), while the other 28 were reasonably informative (0.25 $\leq$ PIC < 0.50) or slightly informative (PIC < 0.25). Notably, the difference in polymorphic level between these markers may be largely dependent on the genetic background of *Luffa* samples amplified. Although we developed a set of polymorphic SSR markers based on two *L. cylindrica* genomes, the high polymorphic rate observed in the *Luffa* population provides a reliable guarantee for their utilization. Cluster analysis of 24 *Luffa* samples showed that *L. cylindrica* and *L. acutangula* were separated into two large groups, consistent with the clustering results obtained using other molecular markers such as SRAP (Cui et al., 2012; Wu et al., 2015; Zhu et al., 2016a) and ISSR (Ye et al., 2017; Guo et al., 2020). The huge genetic difference between *L. cylindrica* and *L. acutangula* provides great potential for continuous improvement of the two *Luffa* species. Population structure analysis showed that interspecific hybridization between *L. cylindrica* and *L. acutangula* such as 'Shengyou × Pingguo F_1' (No. 3) or genetic introgression such as 'Changfeng' (No. 23) could be an effective approach to genetic improvement of the *Luffa* species. Among the *L. cylindrica* samples, except that the only one sample with round-shaped fruit 'Pingguo' (No. 2) and the only one sample with wrinkled fruit skin 'Zaozhou No.2' (No. 8) was assigned to group II-1 and group II-2, respectively, the remaining samples showed a relatively close genetic distance, suggesting that fruit shape and skin were typical grouping characteristics. In conclusion, the polymorphic SSR markers developed from two *L. cylindrica* genomes in this study have a high polymorphic rate in *Luffa* species and can be used as primary candidate markers for genotyping *Luffa* species in the future.

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