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Development of SNP Sites in Loquat Using SLAF-seq Technology

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Abstract A group of SNP locis with high specificity and stability were developed by using SLAF-seq sequencing technology, which will provide theoretical basis for loquat molecular assisted breeding, genetic map construction and species evolution. In the present study, 294 loquat accessions were collected, and genomic DNA was sequenced and analyzed according to SLAF-seq. The reference genome of pear was used for the prediction of electronic enzyme. Double digestion with enzymes HaeIII and Hpy166II then SLAF-seq library was constructed. In this study, over 526.63M reads data were generated for the 294 natural accessions, the number of read lengths obtained for each library was in the range of 119,893-9,305,152 reads, with an average of 1,787,581. The sequencing quality value Q30 ranged from 88.26 to 95.67%, with an average of 93.61%. GC content was distributed in the range of 38.79%~42.92%, with an average value of 40.35%. The data volume of 0.16M reads obtained from rice sequencing was as control, and the efficiency of double ended comparison was 91.28%, indicating that SLAF database was basically normal. The result of bioinformatics analysis showed that 623,356 SLAF tags were obtained, of which 123,498 were polymorphic, with a polymorphism rate of 19.81%. A total of 1,604,434 population SNPs were initially called for this set of polymorphic SLAF tags, leaving 95,960 SNPs at MAF > 0.05 and completeness > 0.8 for the further analyses.
Keywords Loquat; SLAF-seq; Natural population; SNP marker

Loquat (*Eriobotrya japonica* Lindl.) originated in China and has a long history of cultivation in China. A variety of loquat genetic resources have been formed in the process of long-term selection and domestication. Up to now, there are 26 species and varieties or forms of loquat plants. More than 20 Eriobotrya resources in China. There are not only common loquat with high cultivation value, rich species and high evolution degree, but also wild type loquat resources with original characters such as *Eriobotrya malipoensis* Kuan*, Eriobotrya prinoides* Rehd. Et Wils*, Eriobotrya obovata* W.W. Smith*, and Eriobotrya bengalensis* (Roxb.) Hook. F.(Lin, 2017)

Loquat is widely distributed in Henan, Anhui, Jiangsu, Hubei, Hunan, Jiangxi, Zhejiang, Fujian, Guangxi, Yunnan, Guizhou, Sichuan, Taiwan, Hainan and Guangdong. Loquat is also distributed in Vietnam, Myanmar, Thailand and Indonesia. Common loquat is formed in the long-term cultivation and breeding of loquat in China, and there are many varieties. In addition to China, common loquat is also cultivated in Japan, the United States, South Africa, New Zealand, Spain and other places. Loquat was first introduced into Japan from the south of China in the Tang and Song Dynasties, and then spread abroad through Japan (Lin, 2019).

Loquat in China has distinct agronomic characteristics and variety characteristics, which is a valuable genetic resource for loquat gene bank in origin, evolution, resource evaluation, variety selection and production. Although RAPD (Fukuda et al., 2016), SSR (Wu et al., 2015), genic SSR (Sun et al., 2018) and genic SNP (Li et al., 2015), rad-snp (Yang et al., 2017) and other markers have been applied in Loquat Germplasm Resources Evaluation, genetic diversity analysis, genetic map construction, variety identification and domestication. However, in the process of genetic evolution from wild loquat to common loquat, there are obvious changes in their agronomic traits, and these important economic traits are mostly quantitative traits. Only on the basis of a

large number of molecular markers, can we find the excellent alleles of related traits and closely link them with important agronomic traits, so as to analyze the genetic evolution relationship of loquat and molecular assisted selection breeding The improvement of important characters plays an important theoretical guiding significance.

In recent years, high-density and accurate markers based on high-throughput sequencing technology provide a new strategy for the study of plant genetic traits. SLAF-seq (specific locus amplified fragment sequencing) sequencing technology is also widely used in sweet potato (Su et al., 2016), cassava (Yu et al., 2018), *Ammopitanthus mongolicus* (Duan et al., 2018), raspberry (Yang et al., 2018), pitaya (Yu et al., 2018). Studies on SNP marker development and evolutionary relationship of several crops, such as grape (Li et al., 2019), Camellia japonica (Liu et al., 2019), ancient tea tree (Geng et al., 2019), laver moss (Li et al., 2019), Forsythia suspensa (Jiang et al., 2020), Perilla (Jiang et al., 2020) and other crops have achieved remarkable results. However, there are few reports on the development of SNP markers in loquat and its related species by high-throughput sequencing. In this study, we developed SNP molecular markers of loquat including related species by SLAF-seq technology, and obtained molecular markers with high coverage rate, which provided theoretical basis for understanding population structure change, genetic evolution and GWAS correlation analysis of important botanical characters of cultivated loquat species.

1 Results and Analysis

1.1 Database Construction Evaluation

According to the prediction of electronic digestion, haeIII + hpy166II was used in this experiment, and 116,171 SLAF tags were predicted according to the defined length of SLAF tags, as shown in Table 1. Using the rice sequencing result as control (data volume 0.16mreads), the double ended comparison was carried out by soap (Li et al., 2009) software. The results showed that the efficiency of double ended comparison in this experiment was 91.28% (Table 2), which was basically normal. In this study, the residual restriction sites in the inserts of reads were used to reflect the enzyme digestion efficiency. In this study, the enzyme digestion efficiency was 91.82%, and the proportion of partial enzyme digestion was 8.18% (Table 2), indicating that the establishment of SLAF database in this study was normal. In addition, it can be seen from Figure 1 that the length of the control insert is within the expected range, indicating that the sequencing method used in this study has high accuracy and normal sequencing quality.

Table 1 Statistic results of enzyme-cut prediction

Enzyme	Insert Size (bp)	SLAF NumberSLAF
HaeIII+Hpy166II	314-364	116,171

Table 2 The alignment results between obtained reads of control and its genome sequences

Sample	Paired-end mapped reads(%)	Single-end mapped reads(%)	Unmap reads(%)	Digestion Normally(%)	Digestion Partly(%)
Control	91.28	1.29	7.43	91.82	8.18

1.2 Quality assessment of sequencing data

In this study, the analysis range of 125 bp×2 and data evaluation were used to ensure the analysis quality. Through Illumina High-seq 2500 platform sequencing, a total of 526.63M reads data were obtained, and the reading length range of each sample was 119,893-9,305,152, and the average reading length was 1,787,581.

1.2.1 Sequencing quality value distribution check

In this study, Q30, the percentage of bases whose sequencing quality value is greater than or equal to 30, is used to ensure the sequencing quality. Figure 2 shows that the first 125 bp and the last 125 bp represent the distribution of the mass values of the first and the other end of the sequenced reads respectively; the darker the color of the quality values at the same position, the higher the proportion of the mass values in the data. In this study, the distribution range of Q30 sequencing quality value ranged from 88.26% to 95.67%, with an average

of 93.61%. If it is higher than 88%, it indicates that the sequencing base error rate is low and the sequencing data obtained are qualified.

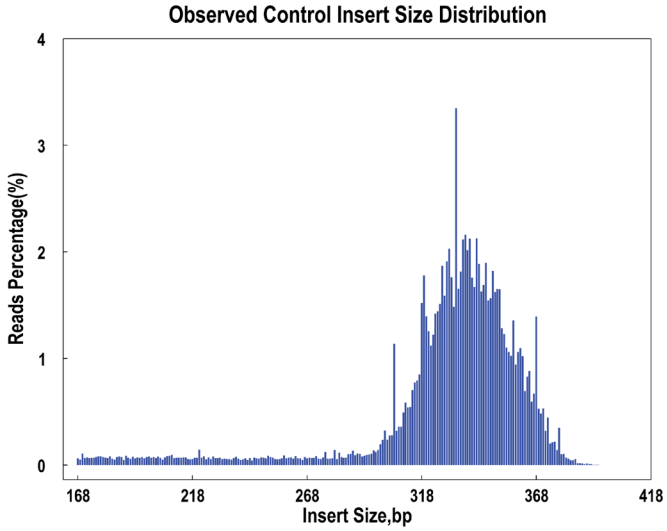

Figure 1 Distribution of insert fragment of control reads

Note: The abscissa is the length of insert fragment, and the ordinate is the reads percentage of corresponding length

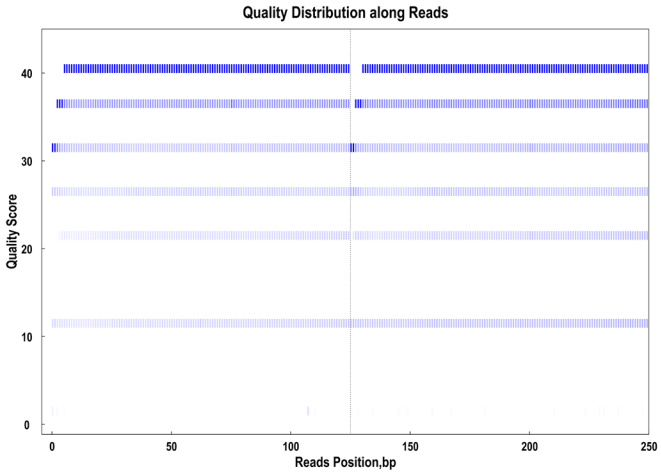

Figure 2 Distribution of sequencing quality values representative sample

Note: The abscissa is the position of Reads, and the ordinate is the quality score of the single base

1.2.2 Base distribution check

The base distribution of SLAF-seq sequenced reads was affected by PCR amplification and restriction sites. The first two bases showed base separation consistent with the restriction site, and the subsequent bases showed varying degrees of fluctuation (Yang et al., 2018). Therefore, we can detect the separation of GC and at by base distribution inspection, and then judge the quality of sequencing. The distribution of sequencing bases is shown in Figure 3. The first 125 bp and the last 125 bp are the base distribution of the first end and the other end of the double ended sequencing sequence, and the single G and C contents fluctuate in the range of 15%~20%. The GC content analysis of the obtained sequences showed that the percentage distribution of G and C bases in the total base was 38.79-42.92%, with an average of 40.35% (Figure 4). The GC content was generally not high, indicating that the sequencing requirements were met.

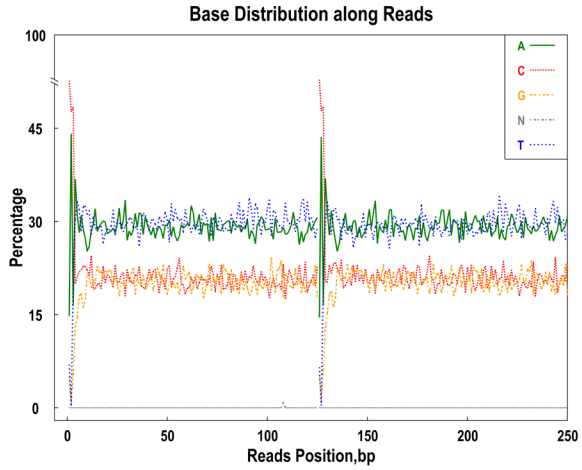

Figure 3 The base content distribution of double terminal 125 bp
Note: The abscissa is the position of Reads , and the ordinate is the quality score of the single base

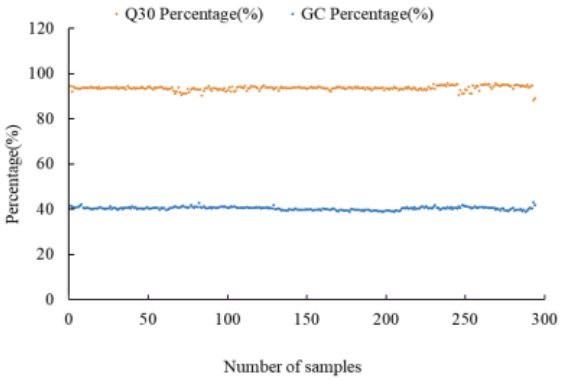

Figure 4 Distribution of Q30 sequencing quality values and GC content
Note: The abscissa is the position of Reads , and the ordinate is the quality score of the single base

1.3 SLAF tag and SNP site statistics

In this study, the number of SLAF tags contained in the corresponding samples ranged from 78,764 to 163,817, and an average of 103,248 SLAF tags were developed for each sample. A total of 623356 SLAF tags were obtained. Among them, 123,498 were polymorphic, and the polymorphism ratio was 19.8%. The total sequencing depth of samples in SLAF tags ranged from 650,577 to 5,594,798, with an average total depth of 1,285,202; the average sequencing depth was 6.19 × - 38.58 × and the average sequencing depth was 12.27 × (Table 3)。

Table 3 Statistics of the SLAF number, total depth and average depth of obtained by sequencing of samples

Type	SLAF number	Total depth	Average depth
maximum	163,817	5,594,798	38.58×
minimum	78,764	650,577	6.19×
average	103,248	1,285,202	12.27×
Total	623,356	377,849,267	3609

In this study, GATK (McKenna et al., 2010) and samtools (Li et al., 2009) were used to compare the sequenced reads to the SLAF tag sequence with the highest depth, so as to obtain a reliable SNP marker dataset. A total of 123,498 polymorphic SLAF tags were obtained, and SNP markers were further developed. Filtration based on integrity (> 0.8) and minor genotype frequency (MAF > 0.05), a total of 95,960 SNPs were obtained. The average integrity of SNPs detected in the samples ranged from 18.5% to 98.53%, with an average of 90.75%; the heterozygosity rate of SNPs in samples ranged from 4.51% to 35.98%, with an average of 19.4%. The specific SNP information statistics are shown in Table 4.

Table 4 Statistics of SNP site points, integrity and heterozygosity obtained by sequencing of different individuals

Type	Total SNPs of population	SNPs in different samples	SNP Integrity	SNP Heter ratio
maximum	-	94551	98.53%	35.98%
minimum	-	17,761	18.5%	4.51%
average	-	87091	90.75%	19.4%
Total	95960	1604434	-	-

2 Discussion and Conclusion

Loquat has been cultivated for 3000 years in China, and its germplasm resources are rich. Abundant germplasm resources are the decisive factors to increase the yield, improve the quality and improve the resistance of loquat. Compared with the conventional methods of RAPD, SSR and genetic SSR, SNP molecular marker technology has the advantages of high efficiency, short time consumption and high coverage. Because of its unique advantages, SNP has become an important auxiliary tool for plant origin and evolution, resource evaluation and assisted breeding.

In recent years, with the rapid development of high-throughput sequencing technology, simple genome sequencing technologies such as SLAF-seq can effectively overcome the problems of genome complexity and lack of sequence and marker information, and are more suitable for the development and analysis of large-scale SNP markers (Li et al., 2018; Yu et al., 2018; Li et al., 2019). For species without reference genome, SLAF-seq technology has been applied very early and achieved the desired results. Such as the development of SNP markers on chromosome 14 of cotton (Chen et al., 2014), the development of specific molecular markers of Elytrigia elongata (Chen et al., 2013), and the development of SNP loci in sweet potato (Su et al., 2014), In the absence of a reference genome, all of them were developed based on SLAF tags, indicating that this technology has become the focus and trend of research and application in many fields such as plant molecular assisted breeding.

In this study, a total of 526.63Mreads data were obtained from 294 loquat specific molecular markers based on high-throughput sequencing. The average reading length was 1787581, the average Q30 and GC contents were 93.61% and 40.35% respectively. The efficiency of double ended comparison was 91.28%. A total of 623,356 SLAF tags were obtained, of which 123,498 were polymorphic, with a polymorphism ratio of 19.81%. A total of 1,604,434 SNP markers were developed on the polymorphic SLAF tag. According to the integrity and MAF values, 95,960 highly consistent SNPs were obtained. In this study, the average sequencing depth was relatively high, and a large number of SNPs were obtained, which were combined with leaf traits (Dan et al., 2017), fruit traits (Deng et al., 2009) and other quantitative traits (Chen et al., 2011) of loquat, provided important genetic information and molecular marker assisted breeding markers for loquat resources evaluation and utilization, such as parent selection, heterosis utilization, association mapping analysis and evolution.

3 Materials and Methods

3.1 Plant materials

In this study, 294 loquat leaf samples were collected from Loquat National Germplasm Resources nursery of Fujian Academy of Agricultural Sciences and Yangdu innovation base of Zhejiang Academy of Agricultural Sciences. The samples were frozen in liquid nitrogen and stored in -70℃ refrigerator. The loquat resources used in the experiment were from different regions, of which 95 were from Fujian, 44 from Yunnan, 37 from Zhejiang, 36 from Guizhou, 15 from Jiangsu, 13 from Japan, 12 from Sichuan, 9 from Guangdong, 6 from Anhui and 6 from Fujian Samples were from Guangxi, 5 from Spain, 4 from Hubei, 3 from the United States, 3 from New Zealand, 2 from Hainan, 1 from Hunan, 1 from Jiangxi, 1 from Shanghai and 1 from South Africa. The test materials were divided into cultivated species or local species, wild or semi wild species and related species, of which 244 were cultivated species, (31 were semi wild species), and 19 were related species (Table 5).

Table 5 The information of regional distribution and category of the collected 294 loquat accessions

Origin	Number	Population category		
		Cultivars/Landraces	(Half)Wild species	Related species
Fujian	95	95	0	0
Yunnan	44	14	14	16
Zhejiang	37	37	0	0
Guizhou	36	21	15	0
Jiangsu	15	15	0	0
Japan	13	12	1	0
Sichuan	12	11	0	1
Guangdong	9	9	0	0
Anhui	6	6	0	0
Guangxi	6	5	0	1
Spain	5	5	0	0
Hubei	4	4	0	0
America	3	3	0	0
New zealand	3	3	0	0
Hainan	2	0	1	1
Hunan	1	1	0	0
Jiangxi	1	1	0	0
Shanghai	1	1	0	0
South Africa	1	1	0	0
Total	294	244	31	19

3.2 DNA extraction

The genomic DNA of loquat leaves were extracted by CTAB, and then detected by 1% agarose gel electrophoresis. Then the DNA concentration and purity were detected by Bio-Photometer nucleic acid detector (Eppendorf), so as to ensure that the DNA genome of loquat reached the requirement of building library (OD260/OD280 between 1.8~2.0). According to the determined standard dosage, the dilution shall be carried out according to the following principles: when the concentration of the original solution x is more than or equal to 120 ng/ μL, the diluted volume shall be 15~120 μL; when the concentration of DNA stock solution is 20 ng/μL＜X≤120 ng/μL, the original solution shall be used, and the sample volume shall not exceed 1/2 of the original solution volume. After the sample was diluted, 2μLwas taken for nanodrop test or LabChip DS test. After the sample is diluted, put the sample back into the original box and store it at -20°C for standby.

3.3 Determination and sequencing of enzyme digestion scheme

Since the loquat genome data has not yet been published, this study selected the genome of pear (*Pyrus spp*) as a reference for further prediction. The actual genome size of *Eriobotrya japonica* is about 654Mb, and the GC content is 40.35%; reference species information: the genome size of pear is 508Mb, and the GC content is 37.28%. Download address: ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/315/295/GCA_ 000315295.1_ Pbr_ v1.0/. The prediction of restriction sites mainly depends on the proportion of digested fragments, distribution, the degree of agreement with the specific experimental system, the number of tags and other factors to determine the most suitable enzyme digestion scheme (Davey et al., 2013). After that, the DNA was digested by enzyme, and the 3′ end was added with A, linked joint, PCR amplification, purification, sample mixing, gel cutting, and library construction. After passing the quality inspection, the library was sent to Beijing Baimaike Biotechnology Co., Ltd. for double ended sequencing. Oryza sativa indica was used as the control to evaluate the accuracy and effectiveness of the enzyme digestion experiment http://rapdb.dna.affrc.go.jp/.

3.4 Data statistics and quality evaluation

After sequencing, the obtained sequences were successively de spliced, decontaminated, and low-quality

reading frames were removed to obtain clean sequences. The sequencing quality, base distribution, sequencing data evaluation and enzyme digestion efficiency, fragment selection and sequencing quality of control sample data were evaluated.

3.5 SLAF tag and SNP tag development

According to the sequence differences of polymorphic SLAF tags among different varieties, a genome-wide SNP marker was developed in all loquat populations through Shengxin analysis, and the representative and highly consistent SNPs in loquat natural population were used for population genetic polymorphism analysis (Duan et al., 2018).

Authors' contributions

Li Xiaoying and Zheng Shaoquan were responsible for the design and implementation of the experiment; Li Xiaoying completed data analysis and paper writing; Xu Hongxia, Hu wenshun, Deng Chaojun and Chen Xiuping participated in the selection of experimental materials; Chen Junwei guided the experimental design, paper writing and revision. All authors read and approved the final manuscript.

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