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Analysis of SSR Information of Flower Transcriptome in Loquat

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Abstract In order to provide scientific basis for studying the genetic diversity and molecular marker-assisted breeding of loquat, new molecular markers of loquat were developed by analyzing flower transcriptome data. In this study, MIcroSAtellite (MISA) and Blast2GO were used for SSR searching, screening, identification, and analysis of non-redundant Unigenes, and Primer 3 was used for SSR primer design. A search found that 28 617 Unigenes contained 44 622 SSR loci, and the frequency of occurrence of SSR loci was 47.51%, and the total average distribution distance was 4.87 kb. Mononucleotide and dinucleotide repeats were the main repeat types of SSR in the loquat flower transcriptome that the SSR of mononucleotide and dinucleotide repeats accounted for 52.09% and 32.83% of the total SSR, respectively. The dominant repeat motifs were A/T and AG/CT, which accounted for 50.94% and 26.70% of the mononucleotide repeats and dinucleotide respectively, and the 65.79% length of motif was concentrated in 12~20 bp. Gene Ontology (GO) functional classification showed that 28 617 loquat flower transcriptome Unigenes containing SSR loci were enriched in 51 GO terms of the 3 GO categories, and the biological process category involved the most GO terms (21). Primers for 34 301 SSR sequences were successfully designed, with a success rate of 76.87%. It is shown by the analysis that the flower transcriptome SSR loci had high frequency, high distribution density, and high polymorphism potential and could provide abundant repeat types. These could provide scientific basis for studying quantitative trait loci mapping, molecular marker-assisted breeding, genome evolution, genetic diversity analysis.

Keywords Loquat (*Eriobotrya japonica*); Flower; Transcriptome; SSR; Primer design

The loquat (*Eriobotrya japonica*) is a small evergreen tree of the Maloideae subfamily of the Rosaceae family (Su et al., 2019). Its fruits are soft and juicy, rich in a variety of amino acids and trace elements, and popular among consumers (Yang et al., 2012). Loquat leaves and flowers are also used to treat cough, asthma, and chronic bronchitis (Kikuchi et al., 2014). Loquat has been cultivated for more than 2 100 years (Yang et al., 2018). In addition to China, more than 30 countries have cultivated loquat, including Spain, Japan, Turkey, India, and Pakistan (Liu et al., 2016; Yang et al., 2018). Similar to fruit trees of the Rosaceae family such as apple, pear, and apricot, loquat belongs to the gametophytic self-incompatibility type, but it is worth noting that compared to other self-incompatibility fruit trees of the Rosaceae family, differences in the intensity of self-incompatibility among species of loquat are particularly common (Yang et al., 2018). Therefore, conducting research on the molecular biology of pollination and fertilization in loquat has important potential theoretical value for studying the molecular mechanisms of self-incompatibility or self-incompatibility in Rosaceae. Domestic and foreign scholars have conducted a large number of molecular biology studies using RAPD (Yang et al., 2011), ISSR (Wang et al., 2010), SNPs (Li et al., 2015), AFLP (Wu et al., 2011), RAD-Seq (Fukuda et al., 2019), SSR (Chen et al., 2014) and other molecular markers, which are of great significance for germplasm conservation, genetic improvement and germplasm innovation of loquat (Wang et al., 2010), but the studies on loquat self-incompatibility or self-compatibility mutations have mainly referred to primers developed by apple and pear fruit trees (Yang et al., 2018), and primers from loquat itself are still lacking.

The types of DNA molecular markers currently applied to loquat are mostly RDMs and GTMs, especially RDMs. While the lag in the study of loquat genome sequence and expressed sequence tags (ESTs) has resulted in fewer

markers that can be developed on other plants being carried out on loquat (Long et al., 2013). Studies have shown that genic SSRs, derived from the transcriptome, are considered to be associated with agronomic trait motifs and thus more valuable for molecular marker-assisted selection (MAS) (Xue et al., 2018). In loquat, some genic SSR markers have been developed by researchers using transcriptome sequence data (Li et al., 2014; Zheng et al., 2015; Sun et al., 2018), which has laid a certain foundation for studies on genetic diversity analysis, molecular marker-assisted breeding, and establishment of breeding populations in *Eriobotrya* genus. There are still few studies on the development of genic SSR markers using loquat transcriptome sequence data, and studies on the development of genic SSR markers using loquat flower transcriptome have not been reported yet.

Therefore, this study analyzed the SSR loci in the loquat transcriptome using RNA-Seq technology in order to provide more genic SSR markers for loquat in terms of self-incompatibility or self-compatibility mutation, genetic diversity analysis and germplasm identification, genome evolution, molecular marker-assisted breeding, SSR marker development and QTL localization.

1 Results and Analysis

1.1 Distribution characteristics of SSR loci in the transcriptome of loquat flowers

The three generations of transcriptome of 'Dawuxing' and 'Huangmi' loquat varieties and 'No. 16' superior plant flower obtained 11.30 Gb, 9.49 Gb and 9.49 Gb clean data, respectively. The total length of the loquat flower transcriptome obtained by assembly and splicing was 217.86 Mb, including 93 927 non-redundant genes (Unigenes), of which 26 956 were over 1 kb long. A search of 93 927 Unigene revealed (Table 1) that 28 617 Unigene contained 44 622 SSR loci, and the frequency of SSR occurrence in Unigene was 30.47%; among them, 26 734 Unigene contained only one SSR locus, 1 883 Unigene contained two SSR loci, 4 357 Unigene contained compound SSR as loci, and the frequency of SSR loci occurrence was 47.51%.

Mononucleotide and dinucleotide repeats were the major types of repeats in the loquat transcriptome, accounting for 84.92% of the total SSRs, with mononucleotide repeats accounting for the largest proportion (52.09%); followed by dinucleotide repeats (32.83%); then trinucleotide repeats (13.42%); tetranucleotide, pentanucleotide and hexanucleotide repeats were few in number, accounting for only 0.92%, 0.27% and 0.47%, respectively. In terms of SSR distribution distance, the total average distribution distance was 4.87 kb. In general, among the six nucleotide repeat types, the higher the frequency of SSR loci, the smaller the average distance of their distribution. The average distance of mononucleotide repeats was only 9.35 kb, dinucleotide repeats was 14.84 kb, trinucleotide repeats was 36.29 kb, tetranucleotide repeats was 531.50 kb, pentanucleotide repeats and hexanucleotide repeats were 1 811.51 kb and 1 030.24 kb, respectively (Table 2).

Table 1 The SSR information in loquat flower transcriptome

Type	Statistic
Number of Unigene	93 927
Total length of Unigene sequence (bp)	217 381 567
Number of loci	44 622
Number of Unigene containing SSR loci	28 617
Number of Unigene containing more than 2 SSR loci	1 883
Number of Unigene containing composite SSR loci	4 357

Table 2 Distribution of SSR loci in loquat flower transcriptome

Repeat type	Number	Proportion (%)	Frequency (%)	Average distance (kb)
Mononucleotide	23 243	52.09	24.75	9.35
Dinucleotide	14 649	32.83	15.60	14.84
Trinucleotide	5 990	13.42	6.38	36.29
Tetranucleotide	409	0.92	0.44	531.50
Pentanucleotide	120	0.27	0.13	1 811.51
Hexanucleotide	211	0.47	0.22	1 030.24
Total	44 622	100	47.51	4.87

1.2 Characteristics of SSR repeat motifs in the loquat flower transcriptome

There were 2 585 repeat motifs in 44 622 SSRs of the loquat transcriptome. Among them, there were 905 mononucleotide repeats, with A/T occurring most frequently (22 729, 50.94%); 572 dinucleotide repeats, with AG/CT dominating overwhelmingly (11 912, 26.70%); 356 trinucleotide repeats, with AAG/CTT occurring most frequently (1 373, 3.08%), followed by AGC/CTG (1 052, 2.36%) and AGG/CCT (1 052, 2.36%); there were 16, 15 and 58 tetra-, penta- and hexanucleotide repeats, respectively, with low repetition frequencies of all types of repetitive motifs, all below 0.5%, and 663 complex SSR motifs (Figure 1).

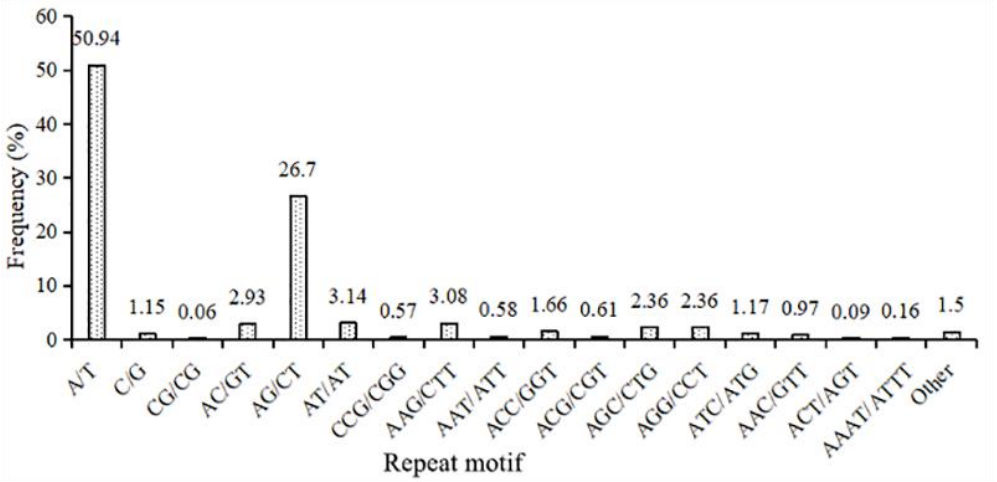

Figure 1 Distribution of different SSR motifs in loquat flower transcriptome

1.3 Number of repeats and motif length of SSRs in the transcriptome of male and female and stamens of loquat flowers

The variation in SSR length was derived from the difference in the number of repeat units, which varied from 5 to 458 repeats and were mostly concentrated in 5~17 repeats, with a decreasing trend in the number of SSRs as the number of repeats increased (Table 3). Repeated primitives had the highest frequency of 10 repetitions, with 7 151 SSRs, accounting for 16.03% of the total SSRs, followed by 6 repetitions (5 156, 11.55%), 11 (4 276, 9.58%), 5 (3 998, 8.96%), 12 (2 815, 6.31%), 7 (2 787, 6.25%), 13 (2 014, 4.51%), 8 (1 904, 4.27%), 14 (1 651, 3.70%), 9 (1 266, 2.84%), 16 (1 179, 2.64%), 17 (1 013, 2.27%), and few with more than 17 replicates. The difference in the frequency of SSR locus repeat units resulted in variation in the length of SSR loci, and the variation of SSR length is an important factor in the variation of its polymorphism. According to the distribution of SSR motif length in the loquat transcriptome (Figure 2), most of the motif lengths were concentrated in 12~20 bp, accounting for 65.79%, followed by 21~30 bp, accounting for 15.57%, then 31~40 bp, accounting for 6.64%, and few motifs above 40 bp, accounting for 12.00%.

1.4 GO functional annotation of target genes at SSR loci

After obtaining the GO annotation information of each gene, all genes were classified by GO function using Blast2GO. 44 622 loquat transcriptome genes containing SSR loci were enriched into 51 GO Term items in three Ontology categories (Figure 3). Of the three Ontology categories, biological process involved the largest number of GO Terms, with 21 (41.18%), while molecular function and cellular component both involved 15 GO Terms, each accounting for 29.41%. Biological process was the most enriched with 75 813 genes, followed by cellular component with 49 132 genes and molecular function with 36 815 genes. Among the 51 GO Terms, metabolic process (19 763), cellular process (16 575), catalytic activity (16 479), binding (15 267), and single-organism process (13 592), cell part (11 644) and cell (11 576) were enriched with more than 10 000 genes.

Table 3 The number of SSR with different repetition types at different repetitions

Repeat number	Repeat type							
	Mononucleotide	Dinucleotide	Trinucleotide	Tetranucleotide	Pentanucleotide	Hexanucleotide	Total	Proportion(%)
5	-	-	3494	256	97	151	3998	8.96
6	-	3646	1350	100	20	40	5156	11.55
7	-	2135	606	32	-	14	2787	6.25
8	-	1631	262	8	-	3	1904	4.27
9	-	1128	231	7	-	-	1266	2.84
10	6148	942	56	4	-	1	7151	16.03
11	3499	743	32	1	-	1	4276	9.58
12	2149	635	31	-	1	-	2815	6.31
13	1475	534	4	-	-	-	2014	4.51
14	1188	456	6	-	-	1	1651	3.7
15	975	418	6	-	-	-	1399	3.14
16	830	343	6	-	1	-	1179	2.64
17	676	337	-	-	-	-	1013	2.27
18	524	264	1	-	-	-	789	1.77
19	452	239		-	-	-	691	1.55
20	353	224	-	-	-	-	577	1.29
21	345	201	-		-	-	546	1.22
22	320	159	1		-	-	480	1.08
23	291	121	-	1	-	-	413	0.93
24	252	102	-	-	-	-	354	0.79
25	276	70	-	-	-	-	346	0.78
26	283	73	-	-	-	-	356	0.8
27	303	44	1	-	-	-	348	0.78
28	307	45	1	-	-	-	353	0.79
29	312	39	-	-	-	-	351	0.79
30	281	19	-	-	-	-	300	0.67
31	198	19	-	-	-	-	217	0.49
32	161	13	1	-	-	-	175	0.39
33	126	13	-	-	-	-	139	0.31
34	103	12	-	-	-	-	115	0.26
35	82	5	-	-	-	-	87	0.19
36	76	12	-	-	-	-	88	0.2
37	52	5	-	-	-	-	57	0.13
38	54	7	-	-	-	-	61	0.14
39	33	2	-	-	-	-	35	0.08
40	38	1	-	-	-	-	39	0.09
41	41	2	-	-	-	-	43	0.1
42	38	3	-	-	-	-	41	0.09
43	37	1	-	-	-	-	38	0.09
44	34	1	-	-	-	-	35	0.08
≥45	931	5	2	-	1	-	939	2.1

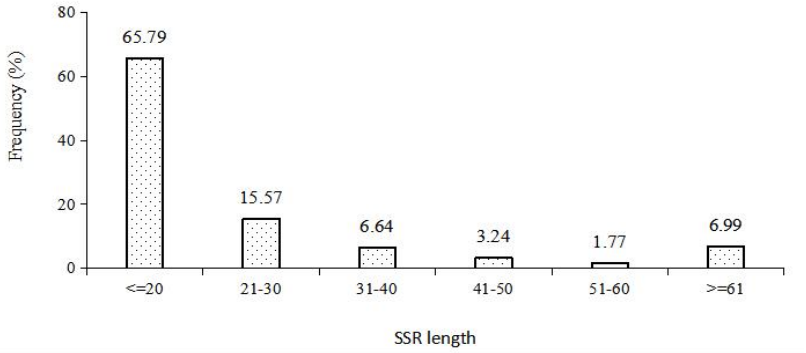

Figure 2 Distribution of SSR length in loquat flower transcriptome

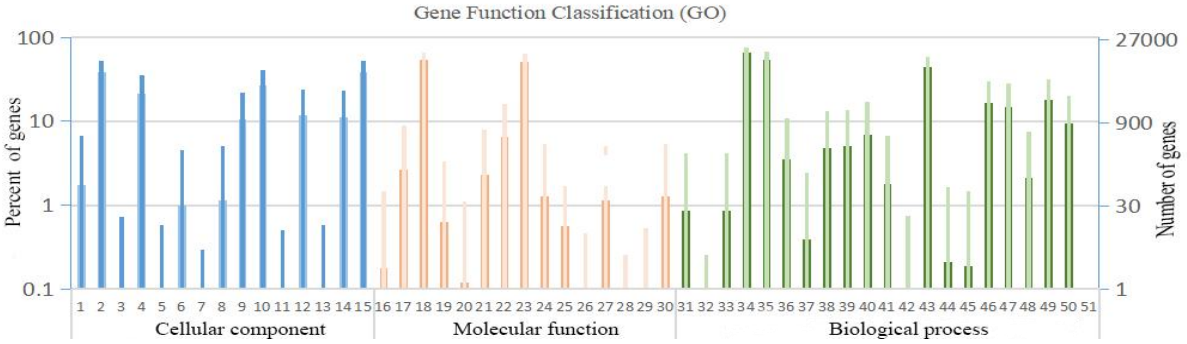

Figure 3 GO Terms of genes containing SSRs

Note: 1: Extracellular region; 2: Cell; 3: Nucleoid4: Membrane; 5: Virion; 6: Cell junction; 7: Extracellular matri; 8: Membrane-enclosed lumen; 9: Macromolecular complex; 10: Organelle; 11: Extracellular region part; 12: Organelle part; 13: Virion part; 14: Membrane part; 15: Cell par; 16: Protein binding transcription factor activity; 17: Nucleic acid binding transcription factor activity; 18: Catalytic activity; 19: Receptor activity; 20: Guanyl-nucleotide exchange factor activity; 21: Structural molecule activity; 22: Transporter activity; 23: Binding; 24: Electron carrier activity; 25: Antioxidant activity; 26: Metallochaperone activity; 27: Enzyme regulator activity; 28: Protein tag; 29: Nutrient reservoir activity; 30: Molecular transducer activity; 31: Reproduction; 32: Cell killing; 33: Immune system process; 34: Metabolic process; 35: Cellular process; 36: Reproductive process; 37: Biological adhesion; 38: Signaling; 39: Multicellular organismal process; 40: Developmental process; 41: Growth; 42: Locomotion; 43: Single-organism process; 44: Biological phase; 45: Rhythmic process; 46: Response to stimulus; 47: Localization; 48: Multi-organism process; 49: Biological regulation; 50: Cellular component organization or biogenesis; 51: Cell aggregation

1.5 SSR primer design for loquat flowers

Primer 3 was applied to design primers for 44 622 Unigenes containing SSR loci, 10 321 sequence primer designs failed and 34 301 pairs of SSR sequence primers were designed successfully, with a success rate of 76.87%. Among the 34 301 SSR primer pairs designed, the most amplification products were expected to be mononucleotide repeats with 13 491 (43.88%), followed by dinucleotide repeats (8 992, 28.92%), then trinucleotide repeats (4 702, 15.12%), and PCR products with complex repeats (containing more than one SSR repeat unit) with 3 403 (10.94%) (Table 4). The length of 34 301 primer pairs ranged from 18 to 27 bp, and the size of amplification products ranged from 100 to 280 bp.

Table 4 Types of repeating primitive contained in expected PCR amplification product

Repeat type	PCR product type	Number	Proportion (%)
Simple repetition	Mononucleotide	13 491	43.38
	Dinucleotide	8 992	28.92
	Trinucleotide	4 702	15.12
	Tetranucleotide	260	0.84
	Pentanucleotide	86	0.28
	Hexanucleotide	164	0.53
Complex repetition	-	3 403	10.94

2 Discussion

Zheng et al. (2015) carried out SSRs site search for 11 798 Unigene with more than 1 kb in the flesh transcriptome of 'Baiyu', 'Jiefangzhong' and 'Huangjinkuai' fruits during the color change stage, and a total of 4 438 SSRs loci were obtained, among which the main repeat types were di-nucleotide repeats and tri-nucleotide repeats, both of which accounted for 68.27% of the total SSRs, while the tetra-nucleotide repeats, penta-nucleotide repeats and hexa-nucleotide repeats types were fewer, accounting for only 1.42%. Li et al. (2014) performed SSRs locus search on 63 608 Unigene in the loquat leaf transcriptome, and a total of 14,004 SSRs loci were obtained, among which dinucleotide and trinucleotide repeat SSRs dominated, accounting for 20.62% and 42.10% of the total SSRs, respectively. In this study, based on the transcriptome data of three generations of loquat flowers, a search of 93 927 Unigene revealed that 28 617 of them contained 44 622 SSRs loci, and the major repeat types of SSRs were different from those of Zheng et al. (2015) and Li et al. (2014), but similar to those research of *Prunus salicina* Lindl. (Conesa et al., 2005), *Lycium ruthenicum* Murr. (Yin et al., 2019) and *Juglans regia* Lindl. (Qin et al., 2019). The dominant types were mononucleotide and dinucleotide repeat SSRs, accounting for 52.09% and 32.83% of the total SSRs, respectively. This may result from the differences in the organs selected for transcriptome sequencing (Zhang et al., 2017) and the differences in factors such as data analysis tools and search criteria (Zong et al., 2016; Fang et al., 2016).

Due to the lower energy of AT bonds compared to GC bonds, AT bonds are prone to fluctuation and breakage (Zhang et al., 2017), the present study is consistent with the distribution trend of the dominant motifs of the transcriptomic SSRs information loci analyzed by previous authors in loquat leaves (Zheng et al., 2015) and fruit flesh (Li et al., 2014), with A/T being the motif with the highest frequency of mononucleotide repeats, AG/CT and AAG/CTT were the dominant motifs for dinucleotide and trinucleotide repeats, which were consistent with the trends of dominant genes for dinucleotide and trinucleotide repeat SSRs in the transcriptome of loquat leaves (Zheng et al., 2015) and fruit pulp (Li et al., 2014), respectively, and also with the dominant genes in *Carya illinoinensis* (Jia et al., 2019), *Cerasus pseudocerasus* (Zong et al., 2016), *Juglans regia* (Qin et al., 2019), *Cerasus pseudocerasus* (Yan et al., 2015), and *Prunus salicina* (Fang et al., 2016) fruit trees were consistent with the distribution trends. The proportion of loquat transcriptome SSRs with more than 12 repeats in this study totaled 40.55%, which indicates that the number of loquat pollen transcriptome SSR repeats is high and it may have good polymorphic potential (Thao et al., 2013; Jia et al., 2019). In addition, Jia et al. (2019) suggested that SSR polymorphism is high when SSRs have motif length greater than or equal to 20 bp and moderate SSR polymorphism with motif length between 10 and 20 bp, and the present study found that 65.79% of SSRs in the loquat flower transcriptome had motif lengths concentrated in 12~20 bp, and 34.21% of SSRs had motif lengths greater than or equal to 20 bp, which indicated that the SSRs obtained in this study had good polymorphism. Primer design was performed for 44 622 Unigenes containing SSR loci, and 34 301 pairs of SSR sequence primers were successfully designed, with a success rate of 76.87%.

In summary, loquat transcriptome SSRs loci can provide abundant repeat types with good polymorphic potential, which can provide an effective theoretical basis for loquat self-incompatibility or self-compatibility mutation studies, molecular marker-assisted breeding, quantitative trait locus localization, genome evolution, genetic diversity analysis, germplasm identification and other studies.

3 Materials and Methods

3.1 Test materials

The materials for this experiment were the flowers of two varieties of five-year old 'Dawuxing' and 'Huangmi' and the superior plant 'No. 16'. The flowers were collected from the Training Base of Kaili College for Excellence in Agriculture and Forestry (26°34′1″N, 107°59′9″E). At the first flowering stage, the flowers that were too large or too small were thinned out and only the large bud stage flowers were kept, then the inflorescences were bagged with nylon nets, and on the third day of the opening of the large bud stage flowers, the pistils and stamens of each variety were collected separately and snap-frozen in liquid nitrogen.

3.2 Data analysis

Biomarker Technologies Co. Ltd., was commissioned to perform the transcriptome sequencing, and the sequencing platform was Illumina Hiseq. The high quality sequences obtained were denovo spliced using Trinity assembly software (Beedanagari et al., 2005) to obtain a sequence containing the least N, which could not be extended at both ends, that was Unigene (spliced non-redundant gene); Unigene assembled from different samples was further sequenced by sequence clustering software to obtain the longest possible non-redundant Unigene (Jia et al., 2019). The MIcroSAtellite (MISA) tool was used for identification, and six types of SSRs were identified by SSR analysis of transcriptomic Unigene sequences: mono-nucleotide repeat SSR, di-nucleotide repeat SSR, triple-base (Trinucleotide) repeat SSR, four-base (Tetra-nucleotide) repeat SSR, five-base (Penta-nucleotide) repeat SSR and six-base (Hexa-nucleotide) repeat SSR; The minimum number of SSR units was set to 10, 6, 5, 5, 5 and 5 repeat sequence, respectively (Zheng et al., 2015). The Blast2GO (http://www.blast2go.de) software (Conesa et al., 2005) was commissioned to perform GO enrichment analysis of the genes containing SSR loci. Primer 3 software was used to design the potential primers for each SSR (Zong et al., 2016).

Authors' contributions

YQ was the experimental designer and executor of the experimental study; FY completed the data analysis and wrote the first draft of the paper; FY participated in the experimental design and analysis of the experimental results; YQ was the conceptualizer and leader of the project and directed the experimental design, data analysis, and paper writing and revision. Both authors read and approved the final manuscript.

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