UDC 575:630
DOI: 10.2298/GENSR1403789J
Original scientific paper

DEVELOPMENT OF 15 GENIC-SSR MARKERS IN OIL-TEA TREE (*Camellia oleifera*) BASED ON TRANSCRIPTOME SEQUENCING

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Jia B., Q.Lin, L. Zhang, X. Tan, X. Lei, X. Hu, and F. Shao (2014*): Development of 15 genic-SSR markers in oil-tea tree (Camellia oleifera) based on transcriptome sequencing*- Genetika, Vol 46, No. 3, 789-797

Oil-tea tree is one of the most important woody edible oil plants; however, lack of useful molecular markers hinders current genetic research. We performed transcriptome sequencing of developing seeds and characterized microsatellites from transcriptome sequences to identify valuable markers for *C. oleifera* molecular genetics research. A total of 69,798 unigenes were identified, in which 6,949 putative SSR motifs from 6,042 SSR-containing unique putative transcripts were discovered. Twenty-nine primer pairs corresponding to 29 unigene loci were designed, of which 15 polymorphic genic-SSR markers were developed in 18 varieties and characterized by capillary electrophoresis. The number of alleles per locus (N_a) ranged from 2 to 14, the expected heterozygosity (H_e) ranged from 0.374 to 0.876, and the polymorphism information content (*PIC*) values ranged from 0.498 to 0.887, respectively. Cross-species amplification was also conducted in 15 varieties of *C. japonica.* All 15 markers successfully amplified PCR products with expected size in *C. japonica* and exhibited polymorphisms. The 15 polymorphic genic-SSR markers will have potential for applications in genetic diversity evaluation, molecular fingerprinting identification, comparative genome analysis, and genetic mapping in the *C. oleifera* and *C. japonica*.

Key words: Camellia oleifera, transcriptome sequencing, unigene, genic-SSR, cross-species amplification

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INTRODUCTION

Oil-tea tree belongs to the Theaceae family and is important native woody plant for production of high quality edible oil, i.e. tea oil in China. Tea oil, extracted from the seeds, is also called ''eastern olive oil'' since the composition of the tea oil is highly similar to olive oil (MA *et al*., 2011). Oil-tea tree includes more than ten species, of which *C. oleifera* is most widely distributed and planted in China and produces the highest yield of oil. To date, a series of authorized elite varieties with high yield have been released for tea oil production. However, more new varieties with new traits such as high quality, multipurpose, and biotic and abiotic stress resistance are required for commercial plantations. Investigating the genetic diversity and relatedness among germplasm resources is a necessary step for breeding and clonal improvements. Simple sequence repeat (SSR) markers are powerful tools for genetic diversity evaluation, molecular fingerprinting identification, and genetic mapping. The newly developed transcriptome sequencing technology including the Roche/454 Genome Sequencer FLX Instrument, the ABI SOLiD System and the Illumina Genome Analyzer not only offers a fast, cost-effective and reliable approach for the generation of large expression-data sets in both model and non-model plants with large and complex genomes (MARIONI *et al*.,2008; MORTAZAVI *et al*., 2008; NAGALAKSHMI *et al*., 2008), but also provides an opportunity to identify and develop unigene-derived genic-SSR markers (GUPTA and GOPALAKRISHNA, 2010; DUTTA *et al*., 2011; ZHANG *et al*., 2012). These new genic-SSR markers are considered better markers than genomic SSR markers because they potentially code for functional proteins and can increase the efficiency of marker-assisted selection.

Recently SSRs were analyzed and developed in *C. chekangoleosa*, a closely related species of *C. oleifera* (WEN *et al*., 2012; SHI *et al*., 2013). Only diploid individuals have been reported in *C. chekangoleosa*, but different ploidy levels (diploids, tetraploids, and hexaploids) occur in *C. oleifera* (HUANG, 2013). *C. oleifera*-derived specific SSR markers have not yet been reported so far, which hinders current genetic research. In our previous work we performed Illumina platform-based transcriptome sequencing of developing seeds of *C. oleifera* to understand the seed fatty acid metabolism (SHAO, 2011). In the present study we characterized microsatellites from the transcriptome sequences and developed polymorphic genic-SSR markers in *C. oleifera*. The novel genic-SSR markers will provide a useful tool for genetic research and comparative genome analysis in *C. oleifera* and other related *Camellia* species.

MATERIALS AND METHODS

Plant materials

Seed samples of an elite authorized variety 'Huashuo' were collected to construct the complementary DNA (cDNA) library at lipid synthesis initiation phase and peak lipid synthesis phase, i.e. 180 and 300 days after flowering (DAF), respectively.

Library preparation, sequencing, and de novo transcriptome assembly

Total RNA was extracted using Micro-to-Midi Total RNA Purification System (Invitrogen) and following the manufacture's protocols. Equal amounts of total RNA from each sample were pooled together. The cDNA library was constructed using the methods described by ZHANG *et al.* (2012) and sequenced on a paired-end flow cell using an Illumina Solexa HiSeq™ 2000 Sequencing System at Beijing Genomics Institute (BGI) in Shenzhen, China. Assembly of integrated high-quality paired-end Illumina reads (>Q20) was determined using the de novo

assembler Velvet and Oases (ZERBINO and BIRNEY, 2008). After removal of all adaptor sequences, empty reads and low quality sequences from the raw reads, the resultant contigs were built into uni-scaffolds based on paired-end information using TGI Clustering (TGICL) tools (PERTEA *et al*., 2003). Finally, the contigs were connected with Trinity, and sequences that could not be extended on either end are defined as unigenes.

Detection of microsatellites and development of genic-SSR markers

The microsatellites detection was conducted using the MIcroSAtellite tool (MISA). The parameters were set for detection of perfect di-, tri-, tetra-, penta-, and hexanucleotide motifs with a minimum of six, five, five, four, and four repeats, respectively. Primer pairs were designed using Primer Premier 5.0. The major parameters for primer design were set as follows: SSR motifs≧ 20 bp, primer length from 18 to 25 nucleotides, PCR product size from 100 to 400 bp, annealing temperature from 52 °C to 60 °C, and GC contents from 40% to 60%.

Validation of genic-SSR primers

Genomic DNA was isolated from young leaves of 18 *C. oleifera* varieties and 15 *C. Japonica* varieties by using a DNA Isolation Kit (Tiangen Biotech, Beijing, China). Out of the 18 *C. oleifera* varieties, three were first used for primers test. PCR reactions were carried out in a total volume of 10 μL containing 1 × buffer, 2 mM of $MgCl_2$, 200 μM of each dNTPs, 0.2 μM of each primer, 0.5 U of *Taq* DNA polymerase (Tiangen Biotech), and 10 ng of DNA template. The PCR was performed on a DNA Engine thermal cycler (ABI9700, USA) under the following conditions: 94 °C for 5 min; 30 cycles of 30 s at 94 °C, 45 s at 56 °C, and 45 s at 72 °C; 10 cycles of 30 s at 94 °C, 45 s at 53 °C, and 45 s at 72 ° C; and a final extension of 5 min at 72 °C. The amplification products were resolved on 2% agarose gels. The loci generating products of the expected size were then assessed for polymorphisms in the 33 varieties with the M13 (-21) (5'-TGTAAAACGACGGCCAGT-3') sequence-tag method (SCHUELKE, 2000). Fluorescently labeled PCR products were analyzed concurrently with the GeneScan™-500LIZ™ Size Standard on an ABI 3730XL sequencer, and sizes were determined with GENEMAPPER version 4.0. Due to the putatively polyploid character of *C. oleifera*, allele frequencies, expected heterozygosity (*H*e) and polymorphism information content (*PIC*) were calculated using the methods described by HUANG (2013). Observed heterozygosity (*H*o) was manually calculated.

RESULTS AND DISCUSSION

Characterization of microsatellites in the C. oleifera unigenes

After the cDNA library was constructed, we sequenced the transcriptome covering two key stages involved in fatty acid biosynthesis in *C. oleifera* developing seeds. Finally 69,798 unigenes were obtained by Trinity with the size ranging from 200 bp to 3,256 bp. Subsequently, the microsatellites were detected with the MISA tool. A total of 6,949 putative SSR motifs from 6,042 SSR-containing unique putative transcripts were identified within the unigenes, that is 9.96% (6,949/69,798) of the unigenes contained at least one of the considered SSR motifs. The SSR motifs were mostly dinucleotide (4,505, 64.83%) and trinucleotide (1,813, 26.09%), and less hexanucleotide (345, 4.96%), pentanucleotide (181, 2.60%) and tetranucleotide (105, 1.51%).

Development of C. oleifera polymorphic genic-SSRs

Considering the large number of dinucleotide SSR motifs, the unigenes containing

dinucleotide motifs were selected to design primers in this study. Twenty-nine primer pairs corresponding to 29 unigene loci were designed for PCR trials. These primers were initially tested for amplification with unlabelled primers on three *C. oleifera* varieties. The amplification products were analyzed by agarose gel electrophoresis (Fig. 1). Seven loci yielded nonspecific products and 7 loci yielded no products. The remaining 15 loci generated products of the expected size, and then were assessed for polymorphism in 18 *C. oleifera* varieties with the M13 (-21) (5'-TGTAAAACGACGGCCAGT-3') sequence-tag method.

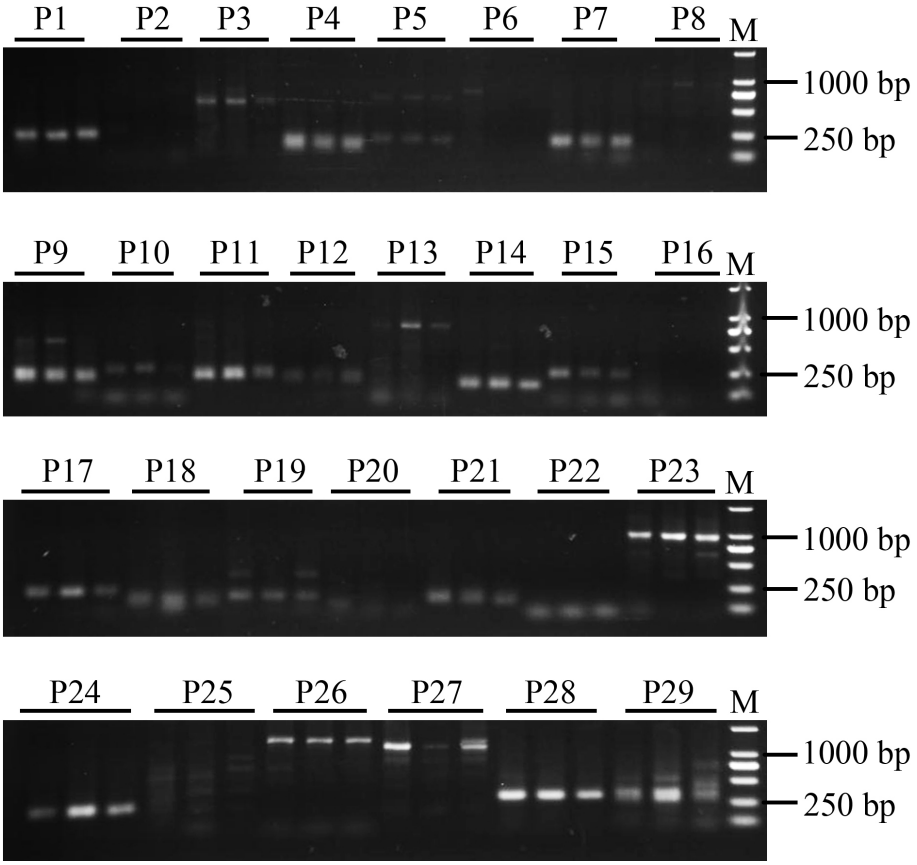

Figure1. Agarose gel electrophoresis of the amplification products with 29 SSR markers.P1 to P29 makers correspond to the unigene locus CoUg8099, CoUg17067, CoUg10255, CoUg9398, CoUg8032, CoUg17435, CoUg1677, CoUg15666, CoUg11169, CoUg11204, CoUg17436, CoUg69448, CoUg17422, CoUg11592, CoUg13753, CoUg12942, CoUg10161, CoUg7784, CoUg7784, CoUg17383, CoUg3417, CoUg7897, CoUg6227, CoUg8134, CoUg68534, CoUg3576, CoUg12179, CoUg16501, and CoUg10140, respectively. M: DL2,000 DNA Marker (Takara, Japan).

Fluorescently labeled PCR products were analyzed concurrently with the GeneScan™-500LIZ™ Size Standard on an ABI 3730XL sequencer, and sizes were determined with GENEMAPPER version 4.0. Capillary electrophoresis is more accurate to estimate the sizes of DNA molecules than agarose gel electrophoresis. The PCR products shown as one band in agarose gel could be clearly separated into four DNA fragments by capillary electrophoresis (Fig. 2).

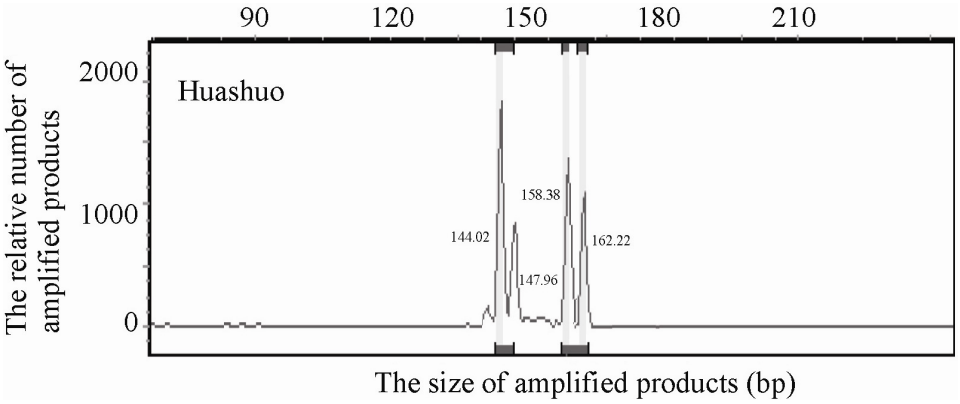

Figure 2. Identification of the number and size of polymorphic genic-SSRs by capillary electrophoresis. The results from CoUg9398 are shown as an example. PCR primers for CoUg9398 locus were used to amplify DNA fragments from genomic DNA of 15 *C. oleifera* varieties. The results from 'Huashuo' producing 4 alleles are presented. The length of the PCR product on the figure is 18 bp longer than the actual size because an 18 bp fluorescent primer M13 (-21) was used for labeling PCR products.

Due to the putatively polyploid character of *C. oleifera*, allele frequencies, expected heterozygosity (*He*) and polymorphism information content (*PIC*) were calculated using the methods described by HUANG (2013). Observed heterozygosity (*Ho*) was manually calculated. Based on capillary electrophoresis, all of the 15 loci displayed polymorphism among the tested *C. oleifera* varieties (Table 1). The number of alleles per locus ranged from 2 to 14 with an average of 9.1. The *He* ranged from 0.374 to 0.876 with an average of 0.746. The *PIC* ranged from 0.498 to 0.887 with an average of 0.782. Out of the 15 loci, 14 were high polymorphic (*PIC* > 0.5), and only one (CoUg11204) was moderate polymorphic (0.25 < *PIC* < 0.5) (Table 2).

Cross-species amplification of the C. oleifera genic-SSRs and putative function of the SSRs containing unigenes

Cross-amplification tests were performed in 15 varieties of *C. Japonica*, a diploid species in *Camellia*. All 15 loci successfully amplified PCR products with expected size in *C. japonica* and exhibited polymorphisms. Allele number ranged from 3 to 6 with an average of 4.7 per locus. *He* ranged from 0.365 to 0.859 with an average of 0.671. *PIC* ranged from 0.354 to 0.832 with an average of 0.650. Thirteen out of the 15 loci were high polymorphic and two (CoUg9398 and CoUg1677) were moderate polymorphic among the 15 *C. japonica* varieties (Table 2). These results indicated that the 15 loci harbored rich polymorphisms in the *Camellia* plants.

Table 1. Characteristics of 15 polymorphic genic-SSRs developed from C. oleifera.

Locus	Primer sequence (5'-3')	Repeat	Ta (°C)	Size (bp)	GenBank reference no.	Putative function
CoUg8099	F: TGGGGATTGCTCAAAAGTGT R: AGGGTGGCTGTGCTGGTATT	(AC)12	57.8 59.4	222	XP_002268591.1	cleavage and polyadenylation specificity factor subunit 2
CoUg9398	F: GTCAATAAGAAAGTATCAGC R: AGTAAGCATGTTAGGTGTC	(GA)11	44 47	124	EOY17366.1	MUTL isoform 1
CoUg1677	F: CAAAGTTGGTGGTGAGAGAT R: GGGATGGTCAAAAGTGGAAT	(CT)12	51.9 56.2	172	EOY17769.1	Kinase superfamily protein isoform 4
CoUg11169	F: GTCTGGTGGCGTTGCTTGCT R: GTCTGCTGATCCGATGGCTG	(CT)16	63.3 61.1	203	XP_002276310.1	transmembrane protein 87B
CoUg11204	F: AACTAGGAAGTGAGACCCC R: CCATTCGACCAGCTGAGGA	(GA)12	51.4 58.4	256	NP_566752.1	AAA-type ATPase family protein
CoUg17436	F: TTGAGGGTGAAGTCGATGA R: AAGGAGTTGGTGAGTAGCA	(TC)40	53 50.1	218	AEC32109.1	ABC transporter family protein
CoUg69448	F: TTTATTGCTACATACGCCCA R: TTTTTCATTTCTTTTTCCCC	(GA)11	54.7 54.3	173	XP_002523791.1	catalytic, putative
CoUg11592	F: TACCGTGCTTGTGTATTCC R: GTGTTTTGTGTTGCTGCCT	(AC)12	51.2 54.2	161	XP_002301775.2	Pantoate--beta-alanine ligase family protein
CoUg13753	F: CACATCATTAGGGTCGTTG R: GGTTTTCACTCTTCAGCAG	(GA)10	51.2 50.1	228	EOY21524.1	3,4-dihydroxy-2-butanone kinase, putative
CoUg10161	F: ATGCTATTTGAGGGTCTTG R: ACTTGGAGTTGGAGTTGTC	(CT)13	50 50	200	ERM96333.1	hypothetical protein AMTR_s00001p00207300
CoUg7784	F: AGAATCATTTTCACCACACC R: TTCCTCATAGTCCCTACCAC	(GA)12	51.8 51.3	111	XP_002280120.2	endoglucanase 16-like
CoUg3417	F: CGGGAATCAAAAAGCTAGG R: AGTGGGTGGTGAACACCAA	(AG)13	54 54.5	152	XP_002264875.1	bidirectional sugar transporter SWEET15
CoUg8134	F: CCAGAGCCAGGAGGAAGTA R: GAGAGAGGGGGTAGAATGA	(AG)10	53.2 50	212	XP_004498870.1	PREDICTED: NIPA-like protein 2-like
CoUg16501	F: TCATCCAATCCAACCAAAC R: CTCAACGATCAAAACCCTG	(AT)10	53 52.1	210	EOY05562.1	Nuclear transcription factor Y subunit C-9 isoform 1
CoUg10140	F: ATAAAGCAAGCAAAACCACACC R: CATAAGAGGAAAATCAGGGCG	(CT)12	59 59.3	272	EOY27178.1	Hydroxyproline-rich glycoprotein family protein

Co and Ug under " Locus" column represent the abbreviation of Oil-tea tree (*C. oleifera*) and unigene followed by the unigene number

Table 2. Genetic parameters generated among 18 C. oleifera varieties and 15 C. Japonica varieties using 15 genic-SSR markers.

Locus	*C. oleifera*				*C. japonica*			
	N_a	H_o	H_e	*PIC*	N_a	H_o	H_e	*PIC*
CoUg8099	3	0.267	0.540	0.609	5	0.125	0.665	0.645
CoUg9398	12	0.889	0.857	0.871	3	0.250	0.365	0.354
CoUg1677	14	1.000	0.876	0.887	4	0.188	0.464	0.449
CoUg11169	12	0.938	0.858	0.871	5	0.375	0.730	0.707
CoUg11204	2	0.154	0.374	0.498	4	0.438	0.732	0.709
CoUg17436	7	0.765	0.697	0.738	8	0.500	0.859	0.832
CoUg69448	13	1.000	0.862	0.875	5	0.063	0.700	0.678
CoUg11592	3	0.600	0.582	0.656	4	0.000	0.581	0.563
CoUg13753	10	0.812	0.823	0.843	4	0.000	0.653	0.633
CoUg10161	13	0.944	0.876	0.887	5	0.250	0.675	0.654
CoUg7784	9	0.667	0.759	0.790	5	0.250	0.734	0.711
CoUg3417	14	1.000	0.868	0.880	5	0.188	0.796	0.771
CoUg8134	10	0.833	0.850	0.865	4	0.375	0.740	0.717
CoUg16501	4	0.467	0.551	0.625	3	0.000	0.613	0.594
CoUg10140	10	0.778	0.812	0.834	6	0.375	0.758	0.734
Mean	9.1	0.741	0.746	0.782	4.7	0.225	0.671	0.650

N_a, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity. *PIC*, polymorphism information content.

To determine the function of polymorphic SSR-associated unigenes, genic-SSRs were evaluated for connections with genes with known functions. The 15 sequences were blasted against the GenBank nonredundant database using BLASTX with an *E*-value $< 1 \times 10^{-5}$. All of the 15 sequences showed significant similarities to known genes, including cleavage and polyadenylation specificity factor subunit 2, Kinase superfamily protein isoform 4, and AAA-type ATPase family protein (Table 1). These informative genic-SSR markers will be valuable for genetic variation analysis and marker-assisted selection in *C. oleifera* and *C. japonica* breeding programs.

ACKNOWLEDGMENTS

This study was supported by the "948" Project of China State Forestry Administration (2012-4-42), the National Natural Science Foundation of China (31100497), and the Key Project of Chinese Ministry of Education (212126).

Received May21th, 2014
Accepted September 05th, 2014

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RAZVOJ 15 SSR MARKERA ZA DRVO ULJANI-ČAJ (*Camellia oleifera*) NA OSNOVU TRANSKRIPCIONOG SEKVENCIONIRANJA

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Izvod

Uljano čajno drvo je jedno od najvažnijih jestivih drvenastih uljanih biljaka.; ali nedostatak korisnih molekualrnih markera ograničava genetička istraživanja. Urađeno je tranaskripciono sekvencioniranje klijanaca i okarakterisani su mikrosateliti iz transkripcionih sekvenci u cilju identifikovanja validnih amrkera za *C. oleifera.* Ukupno 69,798 pojedinačnih gena je identifikovano, od kojih 6,949 pretpostavljenih SSR motiva iz 6,042 SSR-sadržanih jedinstvenih transcript je otkriveno. Dvadeset devet prajmer parova koji odgovaraju 29 jedinstvenim lokusima su dizajnirani, od kojih 15 polimorfnih SSR markera je razvijeno u 18 varijiteta i okarakterisano kapilarnom elektroforezom. Broj alela po lokusu (N_a) je bio u opsegu od 2 do 14, očekivana heterozigotnost (H_e) od 0.374 do 0.876, i *PIC* vrednsot od 0.498 do 0.887. ukrštena amplifikacija u 15 vrsta *C. japonica* je urađena. Svih 15 markera su uspešno amplifikovali PCR produkte sa očekivanim veličinama u *C. japonica.* 15 polimorfnih SSR markera ima potencijal za amplifikaciju u ispitivanju genetičkog diverziteta, molekualrnom fingerpritingu, komparativnoj genomskoj analizi i genetičkom mapiranju *C. oleifera* I *C. japonica*.

Primljeno 21. V. 2014.
Odobreno 05. IX. 2014.