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DEVELOPMENT AND CHARACTERIZATION OF NINETEEN NEW MICROSATELLITE MARKERS (SSRs) FOR *CINNAMOMUM CAMPHORA* (LAURACEAE) BASED ON TRANSCRIPTOME SEQUENCING

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Abstract

Cinnamomum camphora (Lauraceae) is an evergreen tree species with many essential oils that have been used in the fragrance, medicine and chemical industry. However, limited variation of existing microsatellite markers of *C. camphor* was found, which may hinder its applications in genetic diversity. In this study, newly developed microsatellite makers with di- or trinucleotide motifs were isolated using the next-generation transcriptome sequencing approach. Nineteen polymorphic SSR loci of *C. camphor* were developed and characterized. The number of alleles (*Na*) varied from 1 to 7 per polymorphic locus for each population, and the observed and expected heterozygosities (*Ho*, *He*) ranged from 0.000 to 1.000 and from 0.000 to 0.964 respectively. Moreover, four microsatellites loci Cc-44459, Cc-94, Cc-68 and Cc-12 exhibited significant deviation from the Hardy-Weinberg equilibrium. Cross-species amplification showed that most of loci had the transferability in *C. parthenoxylon* and *C. kanehirae*, except for *C. burmannii*. The 19 newly developed microsatellite markers will be useful for exploring the population genetics of *C. camphor* and other species from the same genus.

Key words: *Cinnamomum camphora*; Genetic diversity; Population genetics; RNA-Seq; SSR markers.

Introduction

The *Cinnamomum camphora* is an evergreen arbor species, and widely distributed in eastern Asia, Pacific Islands to Oceania (Kameyama *et al*., 2017). All parts of camphor trees, such as roots, stems, leaves, branches and fruits, contain essential oil, which could be used as bacteriostatic, insecticidal, antioxidant and anti-inflammatory activities (Jiang *et al*., 2016; Fu *et al*., 2016; Zhou *et al*., 2017), and have been used in the fragrance, medicine and chemical industry. Due to its economic importance, camphor trees have been introduced and cultivated in many countries and areas. However, there were few studies on the genetic diversity of *C. camphor*. A few of simple sequence repeat markers had been reported (Kameyama, 2012) but limited variation of those SSRs was found when exploring the genetic diversity of native camphor tree populations in China (Unpublished). Therefore, more SSR primers are necessary to estimate the genetic diversity and population structure of *C. camphor* comprehensively and accurately.

Due to the rich content, wide distribution, high polymorphism and co-dominant inheritance in the whole genome, microsatellite markers (Simple Sequence Repeats, SSRs) have been applied widely in areas of genetic diversity, including constructing genetic maps, analyzing genetic relationships and population structure (Guichoux *et al*., 2011; Lopez *et al*., 2015). Previously there are many studies where utility of SSR markers has been reported to estimate the genetic diversity of different crops (Rabbani *et al*., 2010; Turi *et al*., 2012; Shah *et al*., 2015). Increasingly, with the rapid development of high-throughput sequencing technology, RNAseq data has become one of the most efficiency tool to develop SSR markers (Li *et al*., 2016). 21 polymorphic genic-SSR makers for *C. camphor* were reported, but only tri-, tetra- and hexa-nucleotide motif with limited repeats (5-6 times) were selected and developed (Li *et al*., 2018). Therefore, novel microsatellite markers of *C. camphor* need to be developed based on transcriptome sequencing data, which may provide more available molecular markers for population genetics of *C. camphor*.

Materials and Methods

Plant materials: Leaves of linalool chemotype *C. camphor* were collected from the cutting orchard of Jiangxi Tianxiang Forestry Development Co. LTD, China. Three camphor tree clones were used as biological repeats. All samples were frozen immediately in liquid nitrogen and stored at -80℃ for RNA extraction. Other samples of *C. camphor* were collected from six populations distributed natively in Central and South China (Table 1). In order to avoid collecting the recently cultivated trees, camphor trees with the trunk diameter at breast height of at least 50 cm were selected, and each sample within the population was collected at least 30 m away to avoid multiple individuals from the same clone. Because limited large camphor trees have been reserved, each population included about 10-14 individuals. The fresh leaves were stored in dry silica gel.

RNA/DNA extraction, cDNA library construction and Illumina sequencing: Total RNAs from the linalool chemotype *C. camphor* were extracted from using RNeasy Plant Mini Kit (Qiagen, Hilde, Germany), and

total DNA from other samples was extracted from collecting the silica-dried fresh leaves with a modified CTAB method (Murray & Thompson, 1980). The quality and quantity of isolated RNA/DNA were determined using a Nanodrop and agarose gel electrophoresis. RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). RNA-Seq library was constructed employing the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacture's recommendations, and sequenced on an Illumina HiSeq 4000 sequencing platform. Construction of cDNA library and Illumina sequencing were both conducted by Novogene (Beijing, China). Primer pairs were designed using software Primer3 (Rozen & Skaletsky, 1999).

SSR amplification system and PCR amplification procedure: The amplification reactions were carried out using Mastercycler® nexus (Eppendorf, Germany), and the PCR reactions were performed in a final volume of 25 µl, containing 22 µl of 1.1 × T3 Super PCR Mix (Tsingke, Beijing, China), 1 µl of each primer (0.4 µm) and 1µl total genomic DNA (10-25 ng/µl). All PCRs were conducted with an initial denaturation at 98℃ for 2 min; followed by 30 cycles of denaturation at 98℃ for 10 s, annealing at locus-specific annealing temperature (Table 1) for 10 s, and extension at 72℃ for 10s; and a final extension of 72℃ for 2 min. DNA samples after amplification were stored at 4 ℃ for later use. The PCR products were visualized at 1% agarose gel electrophoresis. Each pair of successful primers that was specially developed with fluorescent dyes (TAMRA, Hex or FAM) at forward primers (5' end) were re-amplified and genotyped (PCR reaction and cycling condition were as above). The PCR products were then separated on ABI 3730xl Genetic Analyzers (Applied Biosystems, USA) to determine the fragment size at each locus, using genotyper 4.0 and LIZ 500 (Applied Biosystems, USA) as an internal size standard.

SSR markers data analysis: In this study, Gene Maker 2.2.0 (SoftGenetics LIC, State College, PA, USA) software was used to analyze the original peak map measured by the biological company, and the peak map was used to determine whether the SSR site was homozygous or heterozygous. Because *C. camphor* is a diploid plant, the single peak stands for homozygous, or bimodal heterozygous. And the low peak value of the heterozygote was required at least half as high as the peak one. Then, the position of each peak was compared with the internal standard of Gene Scan-500LIZ molecular weight in the corresponding panel. The stripe size of each SSR marker was read, and the data were saved in the Microsoft Excel sheets according to the population and locus for further data analysis. Measures of the number of alleles (*Na*), observed value (*Ho*), expected heterozygote (*He*), the inbreeding coefficient within populations (*Fis*), the genetic differentiation (*Fst*) and the gene flow (*Nm*) in each population was found by employing the software Popgene32 (Yeh *et al*., 2002). The polymorphic information content (PIC) value for each locus was calculated using CERVUS version 3.0.3 (Kalinowski *et al*., 2007).

Cross-species amplification: The polymorphic loci isolated from *C. parthenoxylon*, *C. burmannii* and *C. kanehirae* were tested for amplification in 15 individuals of three species, followed the same procedures as above, except that the annealing temperature for each locus was re-optimized.

Results and Discussion

The RNA-Seq of *C. camphor* yielded 24,121,394 clean paired-end reads at least 200 bp in length, and 312,457 unigenes were gained from the clean reads performed by de novo assembly with Trinity (Grabherr *et al*., 2011). MISA (Thiel *et al*., 2003) was used to isolate SSRs, and totally identified 134,851 putative SSRs. 38 primer pairs were selected for further testing based on the region containing at least nine repeats of dinucleotide motifs or six repeats of trinucleotide motifs, a PCR product size with 100-300 bp, a primer length ranging from 20 to 27 nucleotides and annealing temperature with 50-65℃. However, 19 of the 38 primer pairs could amplify and yield polymorphic amplification products among the six sampling populations (Table 2).

Table 1. Locality information for *C. camphor* used in this study.

Population (abbreviation)	Locality	Geographic coordinates	Number of samples	Dates
TR	Tongren, Guizhou, China	27°43′52″N 109°11′05″E	14	March 26,2018
ZH	Zhuhai, Guangdong, China	22°16′25″N 113°34'36"E	13	March 24,2018
HY	Hengyang, Hunan, China	26°58′03″N 112°57′25″E	12	July 1,2017
SM	Sanming, Fujian, China	26°23′28″N 117°20′01″E	12	July 8,2017
NT	Niutian, Jiangxi, China	27°27′24″N 115°40′12″E	13	April 22,2017
HS	Huangshi, Hubei, China	30°20′00″N 115°03′00″E	10	May 3,2017

Table 2. Characteristics of 19 newly SSRs and primer pairs for *C. camphor*.

Locus	Repeat motif	Primer sequences (5′ to 3′)	Size (bp)	Dye	Ta (℃)	GenBank Accession no.
Cc-33551	$(AAG)_7$	F: GGGTCAGATGGAAATGGGGT R: TCCTCTCCTCCTGCTGTCTC	144	HEX	63.3	MN401209
Cc-44459	$(AAG)_6$	F: GGTAACACCATTGCCGGAGA R: ACAAGCTGACCTAAAACCCTT	108	TAMRA	58.5	MN401210
Cc-40436	$(TCC)_7$	F: GGCTTAGATTTTCTTGGCCCC R: TCACATTAAGGGCCCAACAA	102	TAMRA	58.5	MN401211
Cc-42958	$(GGA)_6$	F: GGGTGAAAGGCTCGTAGGAG R: CCCGGTTGTATCAACGGCTA	197	TAMRA	63.4	MN401212
Cc-41285	$(CAG)_6$	F: GGGCAGACGGACCTGAAATT R: TTTCTTGTGAGAGACGCCCC	202	FAM	63.5	MN401213
Cc-34969	$(GAT)_6$	F: GGGGATTAGGCCTTTCGGAG R: CAGTCGCCTAGCCATTCCAA	209	FAM	63.5	MN401214
Cc-85	$(AT)_{10}$	F: AAGGGAAGACTGTGTGCACC R: GGGAATTCAGGTTTGGCTGC	101	FAM	52	MN401215
Cc-20	$(AT)_{10}$	F: AAGGAAGATAATCTCATTCTTCTTTGT R: CAACTCCACCCATCCACCAA	159	TAMRA	49.5	MN401216
Cc-94	$(TA)_{10}$	F: AAAAGCAACCCTCCCGTGAG R: TGTTGGTGAGTCTTGGCATTCT	154	TAMRA	50.5	MN401217
Cc-23	$(TC)_9$	F: AACACCTGTGACTGAGGTCG R: TGTGGTTTCAGCTTGTATTCCTC	211	FAM	50.9	MN401218
Cc-30	$(CT)_{15}$	F: AAACAAGGAGGACAGGGCAG R: TAACACAAAAGGGCACGCAC	103	FAM	52	MN401227
Cc-58	$(AT)_{10}$	F: AACACTTCACGCATTGCACT R: AGGCAATATGGGTGTGTGGT	202	HEX	52	MN401219
Cc-68	$(TC)_{19}$	F: AAATGACCCCAACCGAGCTT R: CCAAAGCAACAGCTACACGG	252	TAMRA	52	MN401220
Cc-05	$(CT)_{13}$	F: AAGCGATCCCCCATCTTCAC R: ACATTACTGTAACCTCCTGCTTCT	106	FAM	51	MN401221
Cc-34	$(TA)_{13}$	F: AATCAGGATGATCAGCGGCC R: CCCCACCCTTGCTCTCATAG	157	HEX	53	MN401222
Cc-12	$(TC)_{16}$	F: AAACGACCAAAGGCAACCAC R: GGCGTCGATTCTCATTTGGC	253	HEX	52	MN401223
Cc-91	$(GA)_{10}$	F: AACTTAAACGTGAGGGCCCC R: CCCTCCCCCATTTTCCCTTC	156	FAM	53	MN401224
Cc-50	$(GA)_{12}$	F: AAAGGTGTCCCCTAGCTTGC R: TGGAAAGCACTAGCCTTGGT	212	HEX	52	MN401225
Cc-45	$(CT)_{19}$	F: AAACCACAGGAGCGTTGGAT R: ACCCTCCAAACTTCACCCTT	263	HEX	52	MN401226

The number of alleles (*Na*) ranged from 1 to 7 in the six populations were evaluated (Table 3). Levels of observed and expected heterozygosity (*Ho*, *He*) ranged from 0.000 to 1.000 and from 0.000 to 0.964, respectively (Table 2). Four microsatellites loci (Cc-44459, Cc-94, Cc-68 and Cc-12) exhibited significant deviation from the Hardy-Weinberg equilibrium. The inbreeding coefficient at total populations (*Fis*) was employed to estimate the neatness of hybrid progenies, generally varying from-1 to 1. *Fis* > 0, reflecting the deletion of heterozygotes; *Fis* < 0, reflecting the excess of heterozygotes. In this study, *Fis* in natural population of camphor tree varied from -0.554 (Cc-34) to 1 (Cc-44459, Cc-42958, Cc-30) with an average value of 0.112. The genetic differentiation coefficient within populations (*Fst*) ranged from 0.042 (Cc-20) to 0.381 (Cc-44459) with an average value of 0.202. The value of gene flow (*Nm*) in camphor populations was from 0.405 (Cc-44459) to 5.667 (Cc-20) with an average value of 1.425, which indicated that there were high levels of the gene flow among populations, and high levels of genetic differentiation. The polymorphism information content (PIC) value ranged from 0.477 to 0.798. The 19 loci were further examined for cross-species amplification in three related species: *C. parthenoxylon*, *C. burmannii* and *C. kanehirae* (Table 4). We found that most of loci can amplify successful in *C. parthenoxylon* and *C. kanehirae*, but only four loci (Cc-33551, Cc-44459, Cc-85 and Cc-45) can amplify successful in *C. burmannii*.

Table 3. Genetic diversity in six *C. camphor* populations based on 19 newly developed polymorphic microsatellite loci.

Locus	TR pop (14)			ZH pop (13)			HY pop (12)			SM pop (12)			NT pop (13)			HS pop (10)			*Fis*	*Fst*	*Nm*	PIC	HW
	Na	*Ho*	*He*	*Na*	*Ho*	*He*	*Na*	*Ho*	*He*	*Na*	*Ho*	*He*	*Na*	*Ho*	*He*	*Na*	*Ho*	*He*					
Cc-33551	2.00	0.250	0.250	3.00	0.500	0.750	4.00	0.250	0.821	3.00	0.667	0.800	4.00	0.250	0.821	3.00	0.500	0.464	0.286	0.263	0.699	0.657	
Cc-44459	1.00	0.000	0.000	3.00	0.000	0.714	2.00	0.000	0.667	2.00	0.000	0.429	3.00	0.000	0.714	2.00	0.000	0.429	1.000	0.381	0.405	0.639	**
Cc-40436	4.00	0.500	0.750	3.00	0.333	0.600	2.00	0.000	0.667	2.00	0.250	0.536	3.00	0.500	0.833	3.00	0.750	0.714	0.309	0.210	0.942	0.703	
Cc-42958	2.00	0.000	0.533	1.00	0.000	0.000	1.00	0.000	0.000	1.00	0.000	0.000	2.00	0.000	0.429	1.00	0.000	0.000	1.000	0.222	0.876	0.547	
Cc-41285	3.00	0.500	0.464	1.00	0.000	0.000	2.00	0.333	0.333	1.00	0.000	0.000	1.00	0.000	0.000	4.00	0.250	0.821	0.228	0.317	0.539	0.571	
Cc-34969	3.00	0.500	0.464	3.00	0.333	0.600	2.00	0.333	0.333	1.00	0.000	0.000	1.00	0.000	0.000	2.00	0.250	0.536	0.143	0.287	0.621	0.630	
Cc-85	2.00	0.667	0.533	2.00	0.000	0.429	3.00	0.000	0.714	4.00	0.750	0.786	3.00	0.500	0.464	3.00	0.500	0.714	0.236	0.255	0.730	0.761	
Cc-20	3.00	0.500	0.607	2.00	0.250	0.250	2.00	0.250	0.250	2.00	0.500	0.429	2.00	0.500	0.429	3.00	0.500	0.464	-0.177	0.042	5.667	0.711	
Cc-94	3.00	0.750	0.679	2.00	0.750	0.536	3.00	0.750	0.679	3.00	0.250	0.607	3.00	0.500	0.464	4.00	0.750	0.750	-0.154	0.077	3.000	0.614	***
Cc-23	2.00	0.667	0.533	2.00	0.000	0.429	1.00	0.000	0.000	2.00	0.333	0.333	3.00	0.000	0.800	2.00	0.250	0.536	0.440	0.196	1.027	0.725	
Cc-30	1.00	0.000	0.000	2.00	0.000	0.429	2.00	0.000	0.533	1.00	0.000	0.000	1.00	0.000	0.000	2.00	0.000	0.571	1.000	0.290	0.612	0.524	
Cc-58	4.00	0.750	0.821	4.00	0.333	0.867	3.00	0.500	0.679	2.00	0.250	0.250	2.00	0.333	0.333	5.00	0.750	0.571	0.111	0.290	0.612	0.764	
Cc-68	2.00	1.000	0.571	2.00	0.667	0.533	3.00	0.500	0.607	4.00	1.000	0.821	2.00	0.667	0.533	2.00	0.667	0.533	-0.460	0.102	2.191	0.523	***
Cc-05	3.00	1.000	0.750	3.00	0.250	0.607	4.00	1.000	0.800	3.00	0.333	0.733	4.00	1.000	0.821	5.00	1.000	0.857	-0.165	0.174	1.185	0.760	
Cc-34	4.00	1.000	0.750	3.00	1.000	0.679	3.00	1.000	0.679	3.00	1.000	0.714	3.00	1.000	0.733	5.00	1.000	0.893	-0.554	0.123	1.778	0.767	
Cc-12	3.00	1.000	0.679	4.00	1.000	0.821	3.00	0.750	0.607	3.00	1.000	0.679	4.00	1.000	0.786	5.00	1.000	0.857	-0.484	0.104	2.163	0.477	***
Cc-91	3.00	0.750	0.607	4.00	0.750	0.821	4.00	0.750	0.750	3.00	0.500	0.464	3.00	0.750	0.536	4.00	0.250	0.750	-0.091	0.240	0.793	0.685	
Cc-50	5.00	1.000	0.857	3.00	1.000	0.679	5.00	1.000	0.933	2.00	1.000	0.571	7.00	1.000	0.964	5.00	0.500	0.893	-0.295	0.123	1.790	0.715	
Cc-45	4.00	0.750	0.786	4.00	1.000	0.750	6.00	1.000	0.929	5.00	1.000	0.857	4.00	0.750	0.821	5.00	1.000	0.893	-0.248	0.147	1.449	0.798	
Average	2.84	0.610	0.560	2.68	0.430	0.552	2.89	0.443	0.578	2.47	0.465	0.474	2.89	0.461	0.552	3.42	0.522	0.645	0.112	0.202	1.425	0.662	

Note: Na =number of alleles; *Ho*=observed heterozygosity; *He*=expected heterozygosity; *Fis*=inbreeding coefficient; *Fst*=genetic differentiation coefficient; *Nm*=gene flow; PIC=polymorphism information content; n=number of individuals; Significant deviation from Hardy-Weinberg equilibrium after Bonferroni correction (**: p<0.01; ***: p<0.001)

Table 4. Cross-species amplification of the 19 isolated loci of camphor.

Locus	*C. parthenoxylon*	*C. burmannii*	*C. kanehirae*
Cc-33551	-	+	-
Cc-44459	+	+	+
Cc-40436	-	-	-
Cc-42958	+	-	+
Cc-41285	-	-	+
Cc-34969	+	-	+
Cc-85	+	+	+
Cc-20	+	-	+
Cc-94	+	-	+
Cc-23	+	-	+
Cc-30	-	-	-
Cc-58	-	-	-
Cc-68	+	-	+
Cc-05	-	-	-
Cc-34	+	-	+
Cc-12	+	-	+
Cc-91	+	-	+
Cc-50	+	-	+
Cc-45	+	+	+

Note: + = Successful amplification; - = Unsuccessful amplification

Conclusions

New nineteen microsatellites primer pairs were developed and proved to be polymorphic in six populations of *C. camphor* in China. These SSRs would be very useful for further investigations of population structure and the assessment of genetic diversity in *C. camphor* and related species.

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Data accessibility

Raw sequences were submitted to the National Center for Biotechnology Information (NCBI). Sequence information for the developed primers has been deposited to NCBI; GenBank accession numbers are provided in Table 1 (MN401209-MN401227).

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