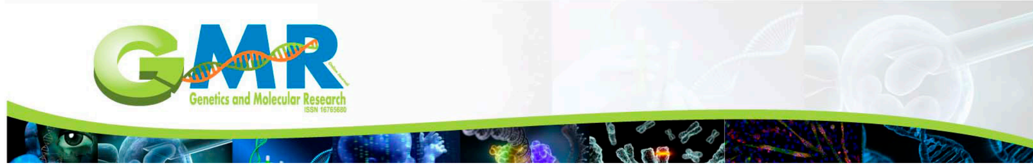

Isolation and characterization of microsatellite markers for *Osmanthus fragrans* (Oleaceae) using 454 sequencing technology

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ABSTRACT. *Osmanthus fragrans* (Oleaceae) is an evergreen shrub or small tree that grows in south China. In this study, Roche 454 FLX+ sequencing combined with the magnetic bead enrichment method was used to isolate microsatellite markers from the genome of *O. fragrans.* A total of 1471 microsatellites that contained enough flanking sequences for primer pair design were identified from 89,633 raw sequencing reads. One hundred primer pairs were randomly chosen to test primer amplification efficiency. Among these tested primer pairs, 20 yielded polymorphic amplification products across 16 individuals from the Albus, Luteus, and Aurantiacus groups. The number of alleles ranged from 2 to 6, with an average of 3.7. The observed heterozygosity ranged from 0 to 0.813, with an average of 0.460. Shannon's information index ranged from 0.463 to 1.707, with an average of 0.975. Six loci (*Of 05*, *Of 06*, *Of 08*, *Of 12*, *Of 15*, and *Of 19*) deviated significantly from Hardy-Weinberg equilibrium ($P < 0.05$), which was due to an excess of homozygotes or heterozygotes. Nine pairs of loci (*Of 01* and *Of 05*; *Of 04* and *Of 05*; *Of 01* and *Of 06*; *Of*

04 and *Of 12*; *Of 02* and *Of 13*; *Of 04* and *Of 13*; *Of 12* and *Of 13*; *Of 04* and *Of 19*; *Of 05* and *Of 19*) showed significant linkage disequilibrium, which indicated significant allelic association between the loci. This set of microsatellite markers will be valuable for molecular marker-assisted breeding in *O. fragrans.*

Key words: Molecular marker-assisted breeding; Microsatellite markers; *Osmanthus fragrans*; Roche 454 sequencing

INTRODUCTION

Osmanthus fragrans (Oleaceae) is an evergreen shrub or small tree that grows in south China (Xu et al., 2014). As an ornamental plant, *O. fragrans* is renowned for its attractive and sweet fragrance, and is regarded as one of the top ten Chinese traditional flowers. This species has been cultivated for more than 2500 years in China. A total of 166 cultivars have been identified, belonging to four groups: Asiaticus, Albus, Luteus, and Aurantiacus (Xiang and Liu, 2008). To date, a few studies have reported on the genetic diversity of *O. fragrans* cultivars using amplified fragment length polymorphism (AFLP; Han et al., 2008; Yan et al., 2009; Yuan et al., 2011), sequence-related amplified polymorphism (SRAP; Li et al., 2009), microsatellite (Zhang et al., 2011; Duan et al., 2013), and inter-simple sequence repeat (ISSR; Fan et al., 2014) markers. However, AFLPs, SRAPs, and ISSRs are dominant markers, which pose difficulties for heterozygosity calculations and paternity analysis. Microsatellites are a type of powerful marker used in molecular marker-assisted breeding for their high polymorphism, ease of genotyping, and co-dominant inheritance (Oliveira et al., 2006). However, the limited number of microsatellite markers for *O. fragrans* (Zhang et al., 2011; Duan et al., 2013) fails to meet the high marker density required in molecular breeding. Thus, it is necessary to develop a set of more powerful markers for molecular marker-assisted breeding in this famous ornamental plant. Next generation sequencing now allows us to obtain a large number of markers in a short time. In this study, we utilized Roche 454 sequencing technology to isolate microsatellite markers for *O. fragrans*.

MATERIAL AND METHODS

Isolation of microsatellite markers

Genomic DNA was extracted from fresh leaves of *O. fragrans* using a plant genomic DNA isolation kit (Tiangen Biotech, Beijing, China), according to the manufacturer protocol. Approximately 2 μg genomic DNA was used to generate a shotgun library, following the 454 Roche protocol. Hybridization of eight 5'-biotinylated oligonucleotides [(AG)10, (AC)10, (AAC)8, (ACG)8, (AAG)8, (ACAT)6, (ATCT)6, and (AGG)8] with the shotgun library was performed to enrich the repetitive motifs. The enriched products were subsequently sequenced using a Roche 454 FLX+ sequencing platform (Roche Applied Science, Indianapolis, IN, USA). Microsatellite identification was performed using MISA (Thiel et al., 2003) with a minimum of five repeats for di-, tri-, tetra-, penta-, and hexanucleotide motifs. Primer pairs were designed using Primer3 (Rozen and Skaletsky, 2000), with default parameters.

Polymerase chain reaction (PCR) amplification and genotyping

Sixteen individuals of *O. fragrans* were collected from the Albus (6 individuals), Luteus (5 individuals), and Aurantiacus groups (5 individuals) in Henan University (Kaifeng, China). PCR amplifications of each sample were performed in a 20-μL reaction volume containing 50 ng genomic DNA, 0.5 μM each primer, and 10 μL 2X Taq PCR MasterMix (0.1 U Taq polymerase/μL, 0.5 mM dNTPs, 20 mM Tris-HCl, pH 8.3) using an S1000 Thermal cycler (Bio-Rad, USA). PCR amplification conditions were set as follows: pre-incubation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 50 s; annealing at a locus-specific annealing temperature for 45 s; elongation at 72°C for 50 s; and a final extension step at 72°C for 8 min. PCR product (5 μL) was mixed with 1 μL 6X loading buffer and then electrophoresed on 8% native polyacrylamide gels. PCR products were visualized using silver staining, and band sizes were reported using a 50 bp DNA ladder (Takara, Dalian, Liaoning, China) as a reference.

Data analysis

Electropherograms for the fragment analysis were analyzed using GeneMarker v1.95 (SoftGenetics, State College, Pennsylvania, USA). The number of alleles per locus (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), and Shannon's information index (SII) were calculated using Popgene 1.32 (Yeh et al., 1997). Linkage disequilibrium (LD) and Hardy-Weinberg equilibrium (HWE) were tested using Fisher's exact tests using GENEPOP 4.2 (Rousset, 2008).

RESULTS AND DISCUSSION

A total of 89,633 raw sequencing reads were yielded using Roche 454 sequencing. These reads ranged from 90 to 589 bp, with an average length of 192 bp. Of these, 1471 sequences were found to contain microsatellites that contained enough flanking sequences for primer pair design and were deposited in GenBank (accession No. KP031709-312207, KP179450-180421). To test primer amplification efficiency, 100 sequences were randomly chosen to design primer pairs, and PCRs were performed. Of the 100 primer pairs, 72 were abandoned because of unsuccessful amplification and non-target amplification, whereas the remaining 28 primer pairs were tested for polymorphisms across 16 individuals from the Albus, Luteus, and Aurantiacus groups.

A total of 20 primer pairs yielded polymorphic amplification products (Table 1). The genetic diversity parameters of each group are presented in Table 2. N_A ranged from 2 (*Of 06*, *Of 18*, and *Of 20*) to 6 (*Of 05*, *Of 08*, and *Of 13*), with an average of 3.7. H_O ranged from 0 (*Of 06*) to 0.813 (*Of 11*), with an average of 0.460. SII ranged from 0.463 (*Of 09*) to 1.707 (*Of 08*), with an average of 0.975. Six loci (*Of 05*, *Of 06*, *Of 08*, *Of 12*, *Of 15*, and *Of 19*) deviated significantly from HWE ($P < 0.05$), which was due to an excess of homozygotes or heterozygotes. Nine pairs of loci (*Of 01* and *Of 05*; *Of 04* and *Of 05*; *Of 01* and *Of 06*; *Of 04* and *Of 12*; *Of 02* and *Of 13*; *Of 04* and *Of 13*; *Of 12* and *Of 13*; *Of 04* and *Of 19*; *Of 05* and *Of 19*) showed significant LD, which indicated significant allelic association between the loci. This set of microsatellite markers has increased the marker density of the *O. fragrans* genome, which will be valuable for molecular marker-assisted breeding of this species.

Table 1. Primer sequences and characterization of 20 microsatellite loci isolated from *Osmanthus fragrans*.

Primer	Primer sequence (5'-3')	Repeat motif	Ta (ºC)	Allele Size (bp)	GenBank accession No.
Of01	F: ATCCACTCTTCCGATGGTCA R: CTGTTTGACGATTTCCGGTT	(AAAAAT)5	58	347-359	KP031709
Of02	F: AAACAGGAACCACAAAACCG R: CCAATCCCATGCTTGAGTCT	(AG)15	58	282-286	KP031710
Of03	F: AAGGGGCCTAGGATCGTTTA R: GTGAGATGGCACCGTAGGAT	(AGG)11	58	284-293	KP031711
Of04	F: CGACGACGCTTACCCTTATT R: CCATTTCTCGCCTATCAAGG	(ATAC)11	58	291-299	KP031712
Of05	F: AAATCCCAACTCACAAGGCA R: TCTGCTGCACTCTTCATTGC	(CA)13	58	208-218	KP031713
Of06	F: AAATGGGTGACTGGGAGTTG R: TGGGCCCTTTTCTGTTCTTA	(GT)8(GA)15(GT)8	58	339-342	KP031714
Of07	F: GCAAAAATGTGAAACGACGA R: TGGCATGTATTCCAGGTTCA	(GTGA)9	60	283-291	KP031715
Of08	F: TGTTGTAACCGCCTTCCAAT R: AGAAACTCCAGGGGCTGATT	(TA)11	60	309-319	KP031716
Of09	F: TTGCCACTAGTTTTCACCCC R: TTGGGGCCACTAGAAACAAC	(TA)8(TG)17	58	240-246	KP031717
Of10	F: TTGGTGACACAATCGACAAT R: AAGGGCAACAGCTTCTGAAA	(TC)6(AC)14	58	335-347	KP031718
Of11	F: CCGCATTAACACCCAAACTT R: CAAAAGAAGACCAATCCCGA	(TC)9(CA)23	58	395-401	KP031719
Of12	F: AATGTGTCATTCAAGGGGGA R: ATGCCTTGTTTGGCTCTCAT	(TCT)14	58	304-312	KP031720
Of13	F: CCATTTTGGTCTTCTCCCAA R: CCAATGAAAAGCCACCATCT	(TCT)5C(CTT)6	58	388-402	KP031721
Of14	F: TGGTCCGCTAAATAACCGAG R: TGGGAGCTCTCCTTCAACAT	(CTT)13	58	313-322	KP031722
Of15	F: CTGAAGCCCGATCCATTAAC R: GCTAGGGCACATATCACCGT	(GT)8(GA)17	60	331-337	KP031723
Of16	F: TGCCACTACCATTTTCACCA R: TCAAACTCCTTGCCACAAAA	(TG)12	60	241-247	KP031724
Of17	F: GGGTGTGCTGTGTTGATGTC R: CCCCTCTCAAATTGCAAGAA	(TG)17(AG)11	60	331-335	KP031725
Of18	F: TTTTGTTTGGCCTTTCTTGG R: AGATCATCGGACGGGTACAG	(TG)21	60	381-383	KP031726
Of19	F: GCATTGCGGATTTTTCATCT R: TTGGGGTATCCAAATCCGTA	(TG)7(GA)15	60	238-244	KP031727
Of20	F: TCGGCAAAAACTTGCAGAC R: ATCCACGTCAATGTGCGATA	(TG)23(AG)9	60	342-344	KP031728

Ta, PCR annealing temperature.

Table 2. Genetic diversity parameters for three groups of *Osmanthus fragrans* assessed using the 20 newly developed microsatellite loci.

Primer	Albus group (N = 6)			Luteus group (N = 5)			Aurantiacus group (N = 5)			Total (N = 16)			
	N_A	H_E	H_O	N_A	H_E	H_O	N_A	H_E	H_O	N_A	H_E	H_O	SII
Of01	2	0.167	0.167	3	0.644	0.800	2	0.200	0.200	3	0.365	0.375	0.615
Of02	2	0.530	0.167	3	0.644	0.800	2	0.467	0.200	3	0.522	0.375	0.786
Of03	3	0.682	0.833	3	0.733	0.800	3	0.644	0.600	3	0.679	0.750	1.086
Of04	3	0.439	0.167	2	0.467	0.600	3	0.511	0.200	3	0.446	0.313	0.743
Of05	4	0.652	0.167	4	0.733	0.600	3	0.511	0.400	6	0.730	0.375*	1.444
Of06	2	0.546	0.000	2	0.356	0.000	2	0.533	0.000	2	0.508	0.000*	0.685
Of07	3	0.667	0.333	4	0.711	0.800	3	0.689	0.800	4	0.700	0.625	1.237
Of08	5	0.742	0.833	4	0.711	0.400	4	0.822	0.200	6	0.831	0.500*	1.707
Of09	3	0.439	0.167	2	0.200	0.200	1	0.000	0.000	3	0.234	0.125	0.463
Of10	4	0.652	0.833	2	0.467	0.600	2	0.556	0.600	4	0.563	0.688	0.955
Of11	4	0.712	1.000	2	0.467	0.600	4	0.644	0.800	4	0.601	0.813	1.086
Of12	3	0.667	0.167	4	0.733	0.600	4	0.733	1.000	5	0.702	0.750*	1.329
Of13	4	0.561	0.167	4	0.733	0.600	4	0.733	0.800	6	0.704	0.500	1.372
Of14	3	0.318	0.333	2	0.356	0.400	2	0.356	0.400	4	0.337	0.375	0.672
Of15	3	0.530	0.333	3	0.511	0.200	3	0.689	0.400	3	0.635	0.313*	1.013
Of16	2	0.409	0.500	3	0.511	0.600	3	0.600	0.800	4	0.478	0.625	0.821
Of17	3	0.727	0.667	3	0.511	0.600	2	0.467	0.200	3	0.605	0.500	0.984
Of18	2	0.303	0.333	2	0.356	0.400	2	0.355	0.400	2	0.315	0.375	0.483
Of19	3	0.621	0.667	4	0.800	0.000	3	0.644	0.400	4	0.748	0.375*	1.337
Of20	2	0.530	0.500	2	0.467	0.600	2	0.467	0.200	2	0.498	0.438	0.676

N, Number of individuals tested; N_A, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; SII, Shannon's information index; *significant deviation from Hardy-Weinberg equilibrium.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Duan YF, Wang XR, Xiang QB, Liang LL, et al. (2013). Genetic diversity of androdioecious *Osmanthus fragrans* (Oleaceae) cultivars using microsatellite markers. *Appl. Plant Sci*. 1: 1200092.

Fan FH, Xia H, Wang ZQ and Chen HG (2014). Analysis on the genetic diversity of *Osmanthus fragrans* Lour. in Xianning with ISSR markers. *J. Hubei Univ.* 36: 272-276.

Han YJ, Dong MF and Yuan WJ (2008). Study on the genetic diversity of *Osmanthus fragrans* cultivars. *Chin. Bull. Bot.* 5: 559-564.

Li M, Hou XL and Hao RM (2009). Analysis of genetic relationships of *Osmanthus fragrans* based on SRAP markers. *Acta Hortic. Sin.* 36: 1667-1775.

Oliveira EJ, Padua JG, Zucchi MI, Vencovsky R, et al. (2006). Origin, evolution and genome distribution of microsatellites. *Genet. Mol. Biol.* 29: 294-307.

Rousset F (2008). Genepop'007: a complete reimplementation of the genepop software for Windows and Linux. *Mol. Ecol. Resour*. 8: 103-106.

Rozen S and Skaletsky H (2000). Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics methods and protocols: methods in molecular biology (Misener S and Krawetz S, eds.) Humana Press, Totowa, USA, 365-386.

Thiel T, Michalek W, Varshney RK and Graner A (2003). Exploiting EST database for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.* 106: 411-422.

Xiang QB and Liu YL (2008). An illustrated monograph of the sweet *Osmanthus* cultivars in China. Zhejiang Science and Technology Press, Hangzhou, China.

Xu YC, Zhou LH, Hu SQ, Hao RM, et al. (2014). The differentiation and development of pistils of hermaphrodites and pistillodes of males in androdioecious *Osmanthus fragrans* L. and implications for the evolution to androdioecy. *Plant Syst. Evol.* 300: 843-849.

Yan XY, Xiao BL, Han YJ, Yuan WJ, et al. (2009). AFLP analysis of genetic relationships and diversity of some Chinese *Osmanthus fragrans* cultivars. *Life Sci. J.* 6: 11-16.

Yeh FC, Yang RC, Boyle T, Ye ZH, et al. (1997). POPGENE: the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada. Available at http://www.ualberta.ca/~fyeh/popgene.html.

Yuan WJ, Han YJ, Dong MF and Shang FD (2011). Assessment of genetic diversity and relationships among *Osmanthus fragrans* cultivars using AFLP markers. *Electron. J. Biotechnol.* 14: 1-9.

Zhang ZR, Fan DM, Guo SQ, Li DZ, et al. (2011). Development of 29 microsatellite markers for *Osmanthus fragrans* (Oleaceae), a traditional fragrant flowering tree of china. *Am. J. Bot.* 98: e356-359.

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