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Authors: Duan, Yifan, Wang, Xianrong, Xiang, Qibai, Liang, Lili, Li, Xuexia, et al.

Source: Applications in Plant Sciences, 1(6)

Published By: Botanical Society of America

URL: <https://doi.org/10.3732/apps.1200092>

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## GENETIC DIVERSITY OF ANDRODIOECIOUS *OSMANTHUS FRAGRANS* (OLEACEAE) CULTIVARS USING MICROSATELLITE MARKERS<sup>1</sup>

YIFAN DUAN<sup>2</sup>, XIANRONG WANG<sup>2,3</sup>, QIBAI XIANG<sup>2</sup>, LILI LIANG<sup>2</sup>, XUEXIA LI<sup>2</sup>,  
YULIAN LIU<sup>2</sup>, AND MENG LI<sup>2</sup>

<sup>2</sup>International Cultivar Registration Center for *Osmanthus*, Nanjing Forestry University, Nanjing, Jiangsu 210037, People's Republic of China

- **Premise of the study:** For cultivar classification, identification, and genetic improvement, microsatellite markers were developed to analyze the genetic diversity of androdioecious *Osmanthus fragrans* cultivars.
- **Methods and Results:** Fifteen microsatellite markers were developed from sequences downloaded from the National Center for Biotechnology Information, which included two with null alleles. These primers were screened on 62 typical androdioecious *O. fragrans* cultivars belonging to four groups (Asiaticus, Albus, Luteus, and Aurantiacus). The number of alleles ranged from two to six, with a mean of 3.7 per locus. The observed and expected heterozygosities ranged from 0.1000 to 0.9091 and from 0.1287 to 0.9167, respectively. Results from structure analyses indicated that Asiaticus and Albus were genetically mixed, and Luteus and Aurantiacus were partially genetically differentiated.
- **Conclusions:** These markers will be useful for genetic study of androdioecious *O. fragrans* cultivars and facilitate cultivar classification, particularly for the cultivar groups Luteus and Aurantiacus.

**Key words:** androdioecy; genetic diversity; microsatellite markers; Oleaceae; *Osmanthus fragrans*.

*Osmanthus fragrans* (Thunb.) Lour. (Oleaceae), a valuable fragrant plant, is found to be functionally androdioecious (the presence of males and hermaphrodites in a population) (Hao et al., 2011). As one of the top 10 traditional flowers in China, *O. fragrans* has been cultivated for about 2500 yr and more than 120 cultivars have been identified. These cultivars are categorized into four groups (Asiaticus, Albus, Luteus, and Aurantiacus) based on morphological and phenological traits (e.g., flower color, peduncle, and flowering period), and there are male and hermaphroditic cultivars in each group (Xiang and Liu, 2008). Moreover, it is thought that the cultivar groups Asiaticus and Albus are less differentiated from wild *O. fragrans* than the other two groups, based on morphological features and research data (Xiang and Liu, 2008).

Several dominant molecular markers have been used for cultivar identification and classification of *O. fragrans* (Xiang and Liu, 2008; Yuan et al., 2011). However, codominant microsatellite markers, which have become preferred markers as they are polymorphic, highly abundant, analytically simple, and transferable, have not been reported in *O. fragrans* cultivars. In this

study, microsatellite markers were developed to analyze the genetic diversity of androdioecious *O. fragrans* cultivars, which will provide new molecular tools for cultivar classification, identification, and genetic improvement.

### METHODS AND RESULTS

Through careful field investigation, *O. fragrans* cultivars and their genders were identified during the 2009 to 2011 flowering seasons. A total of 62 typical *O. fragrans* cultivars (nine hermaphrodites and 53 males) and six closely related species that were used as outgroup taxa were collected (Appendix 1). Genomic DNA was extracted from young and fully expanded leaves of study materials using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). Microsatellite sequences of *O. fragrans* were downloaded from the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/nucleotide>), then analyzed with the Simple Sequence Repeat Identification Tool (SSRIT; <http://www.gramene.org/gramene/searches/ssrtool>) to identify simple sequence repeat (SSR) loci with a minimum length of 10 bp for all repeats. Twenty-nine SSR sequences were selected, and flanking primer sets were designed using the software Primer 5.0 (Clarke and Gorley, 2001). Primers had an optimum length of 22 nucleotides (18 bp minimum, 27 bp maximum) and CG contents ranged from 20% to 80%. The designed primers were synthesized at Genaray Biotech Co. Ltd. (Shanghai, China), and 15 microsatellite markers were selected based on amplification and reproducibility in all accessions (Table 1). The final 12.5- $\mu$ L reaction volume for PCR contained 6.25  $\mu$ L 2 $\times$  Taq Master mix (100 U/mL Taq polymerase, 400  $\mu$ M dNTPs, and 4 mM MgCl<sub>2</sub> [Genaray Biotech Co. Ltd.]), 0.3  $\mu$ M of each forward and reverse primer (Genaray Biotech Co. Ltd.), and 20 ng of DNA template. Amplification was performed with a 5-min initial denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, annealing at 46–52°C for 30 s, and an extension at 72°C for 30 s. A final extension was performed at 72°C for 8 min. A pBR322 DNA-MspI digest marker (Tiangen, Beijing, China) yielding 26 fragments from nine to 622 bp was used as the molecular size standard. PCR products

<sup>1</sup>Manuscript received 29 February 2012; revision accepted 30 September 2012.

The authors are grateful to Dr. Xia Tao, Prof. Hao Ri-Ming, Dr. Xie Chun-Ping, Dr. Hou Bei-Wei, Dr. Wang Nian, and Dr. Ding Guang-Hui for valuable input, data analysis advice, and helpful comments. The authors thank the two anonymous reviewers for their valuable comments on the manuscript. The research was supported by the Doctorate Fellowship Foundation of Nanjing Forestry University.

<sup>3</sup>Author for correspondence: xrworld@163.com

TABLE 1. Characteristics of 15 *Osmanthus fragrans* microsatellite loci.

Locus	$T_a$ (°C)	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	GenBank accession no.
OPF001	50	F: GGAAGCACCACCATAAGC R: AGCAACAGTACCCAGGAG	(TG) <sub>13</sub> (AG) <sub>9</sub>	106–148	GU980659
OPF002	46	F: TTGCATCTTCATTTTACA R: ATGGAAGATAATGAACAA	(CT) <sub>17</sub>	110–190	GU980660
OPF003	50	F: AGTCAGGGGTATCCAGG R: AAGCCCAAAGTATGTTC	(CA) <sub>11</sub> (TA) <sub>8</sub>	123–155	GU980661
OPF004	50	F: CTGCCCTTCTTCTGTGTC R: CACGAACTATCACAATATGTG	(CT) <sub>20</sub>	94–138	GU980662
OPF005	50	F: AACATGATATTCTTGAG R: GTTTTGCCTTAGGGTTAG	(AC) <sub>23</sub>	176–194	GU980663
OPF006	52	F: CCAAAGCCATCACATACC R: CAAGGAGACCTACCCACT	(AG) <sub>8</sub> (AG) <sub>18</sub>	125–170	GU980664
OPF008	50	F: GAGACAGGCATAAACTCT R: TAGCACTCAATCACTTCG	(CT) <sub>17</sub> (GT) <sub>10</sub> GCGT(GC) <sub>6</sub>	76–160	GU980666
OPF016	46	F: TATTCACCAGCAGAGGAG R: AGTTGCTTGTAGAAATGG	(GC) <sub>6</sub> (AC) <sub>10</sub> (AT) <sub>5</sub>	170–188	GU980674
OPF019	52	F: TCAGTGAATGCCTGTGCT R: ACCCTTCTTCTGTGCTT	(AG) <sub>20</sub>	93–117	GU980677
OPF020	48	F: TTGTTTTCTCCCTTCC R: TTCGGTTGTAATGGTTGT	(TC) <sub>11</sub>	111–123	GU980678
OPF022*	46	F: CCTTTCTTCCCTTCTGT R: GAGCCATCGTTGACTTG	(CCT) <sub>5</sub> TCTC(CTT) <sub>16</sub>	136–170	GU980680
OPF023	50	F: TTGGTGGTGC TGGGAAGA R: GTGCCAAACTACCTAACCA	(TC) <sub>11</sub>	82–106	GU980681
OPF024	50	F: CGCACAGAACAGCTCATA R: GGAGAATAATTGGTGGC	(AC) <sub>8</sub>	180–216	GU980682
OPF028	50	F: TAGCTTATGCATTGAGTG R: AAAACCACAGGTAGATGA	(AC) <sub>13</sub>	181–227	GU980686
OPF029*	50	F: CGTCCCTGTTTATGTTGT R: AGGTTAGTGATGCTGCTA	(AG) <sub>14</sub>	200–242	GU980687

Note:  $T_a$  = annealing temperature.

\*Loci OPF022 and OPF029 showed evidence of null alleles.

were separated on 8% denaturing polyacrylamide gels and stained with a silver-staining method.

The presence of null alleles was tested with the program MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004), which suggested loci OPF022 and OPF029 showed evidence of null alleles. These loci were excluded from subsequent data analyses. POPGENE version 1.32 (Yeh et al., 1999) was used to calculate the number of alleles per locus, observed heterozygosity, and expected heterozygosity. The number of alleles ranged from two to six, with a mean of 3.7 per locus. The observed and expected heterozygosities ranged from 0.1000 to 0.9091 and from 0.1287 to 0.9167, respectively (Table 2).

The genetic structure of study materials was inferred using the program STRUCTURE 2.3.1 (Pritchard et al., 2000), with a burn-in length of 30,000 followed by 500,000 cycles, and each run was iterated five times. The number of subgroups ( $K$ ) was determined to be  $K = 4$  using Evanno's method (Evanno

et al., 2005). The results of the structure analyses are presented in Fig. 1. The outgroup (represented in Fig. 1 in yellow) was genetically distinct from all *O. fragrans* cultivar groups. Cultivar groups Luteus and Aurantiacus were somewhat genetically differentiated (mainly represented in Fig. 1 in red and blue, respectively), but gene exchange was evident among many cultivars (indicated in Fig. 1 by the presence of the same color in different groups) and was extensive for Asiaticus and Albus cultivars. The results indicate that Asiaticus and Albus were genetically mixed and incompletely differentiated. Thus, the cultivar groups Asiaticus and Albus possibly have diverged more recently, as they were less genetically differentiated, while the cultivar groups Luteus and Aurantiacus, which displayed greater genetic differentiation, might have diverged earlier. In sum, the molecular results provide some support for the morphological classification of *O. fragrans* cultivars groups Luteus and Aurantiacus (Xiang and Liu, 2008).

TABLE 2. Results of screening of 13 microsatellite loci in *Osmanthus fragrans* cultivars.

Locus	A	Asiaticus group (N = 13)		Albus group (N = 20)		Luteus group (N = 15)		Aurantiacus group (N = 14)	
		$H_o$	$H_e$	$H_o$	$H_e$	$H_o$	$H_e$	$H_o$	$H_e$
OPF001	5	0.8571	0.7582	0.7117	0.8125	0.3571	0.6640	0.9091	0.7316
OPF002	6	0.4545	0.3680	0.4444	0.6016	0.5000	0.6429	0.6429	0.5582
OPF003	6	0.3333	0.3007	0.2632	0.2447	0.5333	0.4667	0.3571	0.4735
OPF004	4	0.8133	0.7645	0.7895	0.6586	0.7857	0.6058	0.8000	0.6737
OPF005	2	0.2500	0.2333	0.2222	0.2032	0.1333	0.1287	0.3571	0.3042
OPF006	3	0.1429	0.3626	0.1508	0.1538	0.2308	0.2185	0.2500	0.2417
OPF008	5	0.8182	0.5411	0.5882	0.4902	0.6000	0.5421	0.3000	0.7053
OPF016	2	0.4000	0.3556	0.2105	0.1935	0.1538	0.1477	0.1667	0.1594
OPF019	3	0.5833	0.5627	0.2778	0.4841	0.5333	0.4805	0.3571	0.3201
OPF020	2	0.1250	0.1854	0.1333	0.1287	0.1000	0.2684	0.2857	0.2637
OPF023	4	0.3333	0.5217	0.6316	0.5249	0.2667	0.2391	0.3571	0.3254
OPF024	3	0.5642	0.8333	0.5917	0.6316	0.4000	0.3425	0.6154	0.4800
OPF028	3	0.5368	0.7273	0.6306	0.4595	0.6667	0.4891	0.5181	0.9167

Note: A = number of alleles;  $H_e$  = expected heterozygosity;  $H_o$  = observed heterozygosity; N = number of cultivars.

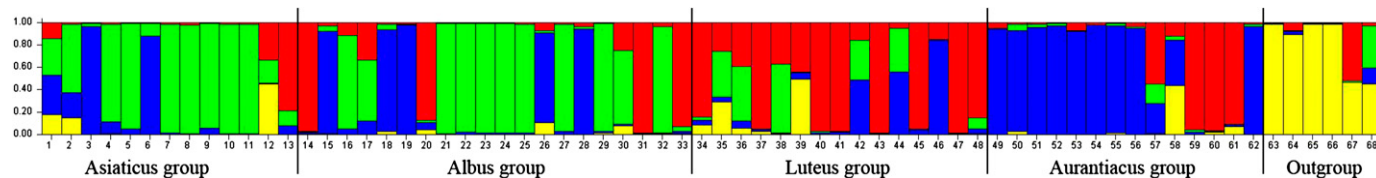


Fig. 1. Estimated genetic structure of study materials for  $K = 4$  obtained with the STRUCTURE program.

### CONCLUSIONS

The 13 microsatellite markers developed for *O. fragrans* are highly polymorphic and informative. These loci will be useful for genetic study of androdioecious *O. fragrans* cultivars and for cultivar classification, particularly for cultivar groups Luteus and Aurantiacus. They also hold potential for further genetic study of *O. fragrans* cultivars.

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#### APPENDIX 1. List of *Osmanthus fragrans* cultivars and outgroup species analyzed in this study.<sup>a</sup>

Code	Cultivar	Accession no.	Gender	Collection site	Geographical coordinates
<b>Asiaticus group</b>					
1	'Danzhuang'	JH004	Hermaphrodite	Jinhua, Zhejiang	29°07'N, 119°39'E
2	'Yuegui'	XN002	Hermaphrodite	Xianning, Hubei	29°50'N, 114°20'E
3	'Daye Sijigui'	LY006	Male	Liyang, Jiangsu	31°26'N, 119°29'E
4	'Tianxiang Taige'	JH001	Male	Jinhua, Zhejiang	29°07'N, 119°39'E
5	'Xiaoye Fodingzhu'	CD002	Male	Chengdu, Sichuan	30°40'N, 104°01'E
6	'Chenghuang Sijigui'	CD004	Male	Chengdu, Sichuan	30°40'N, 104°01'E
7	'Rixianggui'	CD001	Male	Chengdu, Sichuan	30°40'N, 104°01'E
8	'Juye Sijigui'	CQ001	Male	Chongqing	29°35'N, 106°28'E
9	'Daye Fodingzhu'	CD003	Male	Chengdu, Sichuan	30°40'N, 104°01'E
10	'Yuntian Caigui'	CQ002	Male	Chongqing	29°35'N, 106°28'E
11	'Pixian Caigui'	CQ003	Male	Chongqing	29°35'N, 106°28'E
12	'Sijigui'	NJ001	Male	Nanjing, Jiangsu	32°00'N, 118°48'E
13	'Tiannv Sanhua'	JH002	Male	Jinhua, Zhejiang	29°07'N, 119°39'E
<b>Albus group</b>					
14	'Changgengbai'	XN026	Male	Xianning, Hubei	29°50'N, 114°20'E
15	'Baijie'	CZ021	Male	Xianning, Hubei	29°50'N, 114°20'E
16	'Kuoye Zaoyingui'	NJ006	Male	Nanjing, Jiangsu	32°00'N, 118°48'E
17	'Yinsu'	XN005	Male	Xianning, Hubei	29°50'N, 114°20'E
18	'Boye Yingui'	XN034	Male	Xianning, Hubei	29°50'N, 114°20'E
19	'Chiye Yingui'	XN008	Male	Xianning, Hubei	29°50'N, 114°20'E
20	'Juban'	XN037	Male	Xianning, Hubei	29°50'N, 114°20'E
21	'Zie'	XN018	Male	Xianning, Hubei	29°50'N, 114°20'E
22	'Kuoye Ziyingui'	WH003	Hermaphrodite	Wuhan, Hubei	30°37'N, 114°08'E
23	'Yulinglong'	JH006	Male	Jinhua, Zhejiang	29°07'N, 119°39'E
24	'Chuibai'	CZ022	Male	Jinhua, Zhejiang	29°07'N, 119°39'E
25	'Zaoyingui'	NJ004	Male	Nanjing, Jiangsu	32°00'N, 118°48'E
26	'Jiulonggui'	CD005	Male	Chengdu, Sichuan	30°40'N, 104°01'E
27	'Wanyingui'	CZ019	Male	Changzhou, Jiangsu	31°46'N, 119°56'E
28	'Qiuyun'	CZ025	Male	Xianning, Hubei	29°50'N, 114°20'E
29	'Jiangnan Liren'	XN030	Male	Xianning, Hubei	29°50'N, 114°20'E
30	'Yinxing'	XN035	Male	Xianning, Hubei	29°50'N, 114°20'E

APPENDIX 1. Continued.

Code	Cultivar	Accession no.	Gender	Collection site	Geographical coordinates
31	'Ziyingui'	NJ007	Hermaphrodite	Nanjing, Jiangsu	32°00'N, 118°48'E
32	'Liuyegui'	XN005	Male	Xianning, Hubei	29°50'N, 114°20'E
33	'Changye Bizhu'	CZ009	Hermaphrodite	Jinhua, Zhejiang	29°07'N, 119°39'E
<b>Luteus group</b>					
34	'Zijingui'	NJ007	Hermaphrodite	Chengdu, Sichuan	30°40'N, 104°01'E
35	'Susheng Jingui'	CD008	Male	Chengdu, Sichuan	30°40'N, 104°01'E
36	'Changbing Jingui'	CD008	Male	Chengdu, Sichuan	30°40'N, 104°01'E
37	'Wandianjin'	JH008	Male	Jinhua, Zhejiang	29°07'N, 119°39'E
38	'Chuizhihuang'	JH009	Male	Jinhua, Zhejiang	29°07'N, 119°39'E
39	'Congzhongxiao'	JH011	Male	Jinhua, Zhejiang	29°07'N, 119°39'E
40	'Xiaoye Zijingui'	JH012	Hermaphrodite	Jinhua, Zhejiang	29°07'N, 119°39'E
41	'Lihuang'	JH010	Male	Jinhua, Zhejiang	29°07'N, 119°39'E
42	'Yuanban Jingui'	CZ013	Male	Changzhou, Jiangsu	31°46'N, 119°56'E
43	'Zuiyun'	XNQS005	Hermaphrodite	Xianning, Hubei	29°50'N, 114°20'E
44	'Xiaojinling'	JH013	Male	Jinhua, Zhejiang	29°07'N, 119°39'E
45	'Boye Jingui'	NJ009	Male	Nanjing, Jiangsu	32°00'N, 118°48'E
46	'Jinqiugui'	NJ008	Male	Nanjing, Jiangsu	32°00'N, 118°48'E
47	'Qiugui'	XN016	Male	Xianning, Hubei	29°50'N, 114°20'E
48	'Huangchuan Jingui'	XNXY007	Hermaphrodite	Xianning, Hubei	29°50'N, 114°20'E
<b>Aurantiacus group</b>					
49	'Zuijihong'	CZ032	Male	Changzhou, Jiangsu	31°46'N, 119°56'E
50	'Chiye Dangui'	CZ007	Male	Changzhou, Jiangsu	31°46'N, 119°56'E
51	'Suzhou Qiancheng'	CZ009	Male	Changzhou, Jiangsu	31°46'N, 119°56'E
52	'Pingmaihong'	CZ010	Male	Changzhou, Jiangsu	31°46'N, 119°56'E
53	'Yingye Dangui'	CZ014	Male	Changzhou, Jiangsu	31°46'N, 119°56'E
54	'Xionghuanggui'	CD009	Male	Chengdu, Sichuan	30°40'N, 104°01'E
55	'Zhusha Dangui'	CZ017	Male	Changzhou, Jiangsu	31°46'N, 119°56'E
56	'Pucheng Dangui'	PC001	Male	Pucheng, Fujian	27°55'N, 118°32'E
57	'Moye Dangui'	XNQS003	Male	Xianning, Hubei	29°50'N, 114°20'E
58	'Boye Dangui'	CQ004	Male	Chongqing	29°35'N, 106°28'E
59	'Dahua Dangui'	CZ039	Male	Changzhou, Jiangsu	31°46'N, 119°56'E
60	'Hangzhou Dangui'	JH014	Male	Jinhua, Zhejiang	29°07'N, 119°39'E
61	'Xiaoye Dangui'	CZ018	Male	Changzhou, Jiangsu	31°46'N, 119°56'E
62	'Zhuangyuanhong'	CZ003	Male	Changzhou, Jiangsu	31°46'N, 119°56'E
<b>Outgroup</b>					
63	<i>O. cooperi</i>	NJ010		Nanjing, Jiangsu	32°00'N, 118°48'E
64	<i>O. heterophyllus</i> 'Goshiki'	NJ011		Nanjing, Jiangsu	32°00'N, 118°48'E
65	<i>O. heterophyllus</i>	NJ012		Nanjing, Jiangsu	32°00'N, 118°48'E
66	<i>O. fordii</i>	JH016		Jinhua, Zhejiang	29°07'N, 119°39'E
67	<i>O. serrulatus</i>	JH015		Jinhua, Zhejiang	29°07'N, 119°39'E
68	<i>O. armatus</i>	JH017		Jinhua, Zhejiang	29°07'N, 119°39'E

<sup>a</sup>All the cultivars, their genders, and the outgroup species were identified with the help of Prof. Xiang Qi Bai, the international cultivar registration authority for *Osmanthus*. Voucher specimens of all the cultivars and species with their accession numbers were deposited in the herbarium of Nanjing Forestry University (NF).