# Genetic Diversity of Androdioecious Osmanthus fragrans (Oleaceae) Cultivars Using Microsatellite Markers

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Source: Applications in Plant Sciences, 1(6)

Published By: Botanical Society of America

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

Applications in Plant Sciences 2013 1(6): 1200092

PRIMER NOTE

# GENETIC DIVERSITY OF ANDRODIOECIOUS *OSMANTHUS FRAGRANS* (OLEACEAE) CULTIVARS USING MICROSATELLITE MARKERS[1]

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- *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.

[1]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.

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doi:10.3732/apps.1200092

## 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/nuccore), 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 Generay 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-μL reaction volume for PCR contained 6.25 μL 2× *Taq* Master mix (100 U/mL *Taq* polymerase, 400 μM dNTPs, and 4 mM $MgCl_2$ [Generay Biotech Co. Ltd.]), 0.3 μM of each forward and reverse primer (Generay 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

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. |
|---|---|---|---|---|---|
| OFP001 | 50 | F: GGAAGCACCACCATAAGC<br>R: AGCAACAGTACCCAGGAG | $(TG)_{13}(AG)_9$ | 106–148 | GU980659 |
| OFP002 | 46 | F: TTGCATCTTCATTTTACA<br>R: ATGGAAGATAATGAACAA | $(CT)_{17}$ | 110–190 | GU980660 |
| OFP003 | 50 | F: AGTCAGGGGTATTCCAGG<br>R: AAGCCCAAAGTATGTTCC | $(CA)_{11}(TA)_8$ | 123–155 | GU980661 |
| OFP004 | 50 | F: CTGCCTCTTCTTCTGCTC<br>R: CACGAACTATCACAATATGTG | $(CT)_{20}$ | 94–138 | GU980662 |
| OFP005 | 50 | F: AACATGATATTCTTGGAG<br>R: GTTTTGCCTTAGGGTTAG | $(AC)_{23}$ | 176–194 | GU980663 |
| OFP006 | 52 | F: CCAAAGCCATCACATACC<br>R: CAAGGAGACCTACCCACT | $(AG)_8(AG)_{18}$ | 125–170 | GU980664 |
| OFP008 | 50 | F: GAGACAGGCATAAACTCT<br>R: TAGCACTCAATCACTTCG | $(CT)_{17}(GT)_{10}GCGT(GC)_6$ | 76–160 | GU980666 |
| OFP016 | 46 | F: TATTCACCAGCAGAGGAG<br>R: AGTTGCTTGTAGAAATGG | $(GC)_6(AC)_{10}(AT)_5$ | 170–188 | GU980674 |
| OFP019 | 52 | F: TCAGTGAATGCCTGTGCT<br>R: ACCCTTTCTTCTGTGCTT | $(AG)_{20}$ | 93–117 | GU980677 |
| OFP020 | 48 | F: TTGTTTTCTCCCTCTTCC<br>R: TTCGGTTGTAATGGTTGT | $(TC)_{11}$ | 111–123 | GU980678 |
| OFP022* | 46 | F: CCTTTCTTTCCCTTCTGT<br>R: GAGCCATCGTTGTACTTG | $(CCT)_5TCTC(CTT)_{16}$ | 136–170 | GU980680 |
| OFP023 | 50 | F: TTGGTGGTGCTGGGAAGA<br>R: GTGCCAAACTACCTAACCA | $(TC)_{11}$ | 82–106 | GU980681 |
| OFP024 | 50 | F: CGCACAGAACAGCTCATA<br>R: GGAGAATAATTTGGTGGC | $(AC)_8$ | 180–216 | GU980682 |
| OFP028 | 50 | F: TAGCTTATGCATTGAGTG<br>R: AAAACCACAGGTAGATGA | $(AC)_{13}$ | 181–227 | GU980686 |
| OFP029* | 50 | F: CGTCCCTGTTTATGTTGT<br>R: AGGTTAGTGATGCTGCTA | $(AG)_{14}$ | 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.

| | | Asiaticus group ($N = 13$) | | Albus group ($N = 20$) | | Luteus group ($N = 15$) | | Aurantiacus group ($N = 14$) | |
|---|---|---|---|---|---|---|---|---|---|
| Locus | $A$ | $H_o$ | $H_e$ | $H_o$ | $H_e$ | $H_o$ | $H_e$ | $H_o$ | $H_e$ |
| OFP001 | 5 | 0.8571 | 0.7582 | 0.7117 | 0.8125 | 0.3571 | 0.6640 | 0.9091 | 0.7316 |
| OFP002 | 6 | 0.4545 | 0.3680 | 0.4444 | 0.6016 | 0.5000 | 0.6429 | 0.6429 | 0.5582 |
| OFP003 | 6 | 0.3333 | 0.3007 | 0.2632 | 0.2447 | 0.5333 | 0.4667 | 0.3571 | 0.4735 |
| OFP004 | 4 | 0.8133 | 0.7645 | 0.7895 | 0.6586 | 0.7857 | 0.6058 | 0.8000 | 0.6737 |
| OFP005 | 2 | 0.2500 | 0.2333 | 0.2222 | 0.2032 | 0.1333 | 0.1287 | 0.3571 | 0.3042 |
| OFP006 | 3 | 0.1429 | 0.3626 | 0.1508 | 0.1538 | 0.2308 | 0.2185 | 0.2500 | 0.2417 |
| OFP008 | 5 | 0.8182 | 0.5411 | 0.5882 | 0.4902 | 0.6000 | 0.5421 | 0.3000 | 0.7053 |
| OFP016 | 2 | 0.4000 | 0.3556 | 0.2105 | 0.1935 | 0.1538 | 0.1477 | 0.1667 | 0.1594 |
| OFP019 | 3 | 0.5833 | 0.5627 | 0.2778 | 0.4841 | 0.5333 | 0.4805 | 0.3571 | 0.3201 |
| OFP020 | 2 | 0.1250 | 0.1854 | 0.1333 | 0.1287 | 0.1000 | 0.2684 | 0.2857 | 0.2637 |
| OFP023 | 4 | 0.3333 | 0.5217 | 0.6316 | 0.5249 | 0.2667 | 0.2391 | 0.3571 | 0.3254 |
| OFP024 | 3 | 0.5642 | 0.8333 | 0.5917 | 0.6316 | 0.4000 | 0.3425 | 0.6154 | 0.4800 |
| OFP028 | 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.

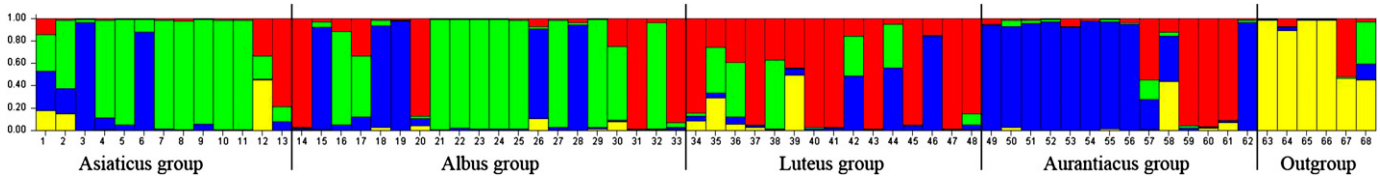


Fig. 1. Estimated genetic structure of study materials for $K = 4$ obtained with the STRUCTURE program.

## 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.[a]

| Code | Cultivar | Accession no. | Gender | Collection site | Geographical coordinates |
|---|---|---|---|---|---|
| **Asiaticus group** | | | | | |
| 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 |
| **Albus group** | | | | | |
| 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 | ‘Chuiban’ | 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 |
| **Luteus group** | | | | | |
| 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 |
| **Aurantiacus group** | | | | | |
| 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 |
| **Outgroup** | | | | | |
| 63 | *O. cooperi* | NJ010 | | Nanjing, Jiangsu | 32°00′N, 118°48′E |
| 64 | *O. heterophyllus* 'Goshiki' | NJ011 | | Nanjing, Jiangsu | 32°00′N, 118°48′E |
| 65 | *O. heterophyllus* | NJ012 | | Nanjing, Jiangsu | 32°00′N, 118°48′E |
| 66 | *O. fordii* | JH016 | | Jinhua, Zhejiang | 29°07′N, 119°39′E |
| 67 | *O. serrulatus* | JH015 | | Jinhua, Zhejiang | 29°07′N, 119°39′E |
| 68 | *O. armatus* | JH017 | | Jinhua, Zhejiang | 29°07′N, 119°39′E |

[a]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).