AFLP analysis of genetic relationships and diversity of some Chinese *Osmanthus fragrans* cultivars[☆]

Xueyan Yan[1,#], Baolin Xiao[1,#], Yuanji Han[1], Wangjun Yuan[1,2], Fude Shang[1,*]

[1]*College of Life Sciences & Institute of Agricultural Biotechnology*, *Henan University*, *Kaifeng*, *Henan* 475001, *China*;
[2]*College of Pharmaceutical Sciences*, *Henan University*, *Kaifeng*, *Henan* 475001, *China*

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Abstract

The genetic diversity and genetic relationships of 46 *Osmanthus fragrans* cultivars, collected from Hubei, Zhejiang, and Guangxi provinces in China, were analyzed by the technique of amplified fragment length polymorphism (AFLP). Ten primer combinations were used and generated 436 scorable bands including 269 polymorphic. It indicated that each primer combination generated 26.9 polymorphic bands. Genetic similarities were obtained using simple matching (SM) coefficients, and a dendrogram of the 46 cultivars was established by UPGMA clustering method. The high level genetic variations in 46 *O. fragrans* cultivars were proved by the SM coefficient value from 0.69 to 0.87. The cluster analysis suggested that the 46 *O. fragrans* cultivars could be divided into five groups, and this result was not absolutely consistent with the morphology-based traditional classification. The cluster analysis showed that there were close genetic relationships among cultivars of the same flower color, and the geographic origin of *O. fragrans* was correlated with the analysis cluster results at a certain level. Cluster analysis also indicated that Guangxi cultivars were distinct from those of Hubei and Zhejiang. [Life Science Journal. 2009; 6(2): 11 – 16] (ISSN: 1097 – 8135).

Keywords: AFLP; *Osmanthus fragrans*; cultivar; genetic diversity; UPGMA

1 Introduction

Osmanthus fragrans (*O. fragrans*), belongs to the Oleaceae family, is cultivated extensively as one of the most valuable of fragrance and ornamental plants in China. A long history of domestication was confirmed by the descriptions of this plant in 2500 years old documents. As *O. fragrans* has been cultivated for a long time, a large number of intraspecific varieties occurred under the influence of both natural and artificial selections. At present, there are about 157 cultivars which could be categorized into 4 cultivar groups (Asiaticus Group, Albus Group, Luteus Group, and Aurantiacus Group) according to morphological and physiological characteristics[1,2].

Gene diversity research is significant to illuminate the evolutionary and classification in cultivars of *O. fragrans* not only in theory but also in cultivar breeding and arrangement in garden. However, no reasonable and accepted taxonomic principles and system are established up to now, and there is still much confusion in cultivar nomenclature. There is still a need for a better genetic diversity assessment and varietal identification by using high throughput marker technologies.

The identification of *O. fragrans* cultivars has been traditionally carried out by morphological and physiological traits[2]. Although these methods are efficient, they present practical drawbacks because of the effect of environmental fluctuations on the expression of most morphological traits.

Several researchers sought to rectify the weakness of the traditional approach by using biochemical markers[3,4]. Although such studies provided some useful insights, they posed their own problems. Biochemical markers are not necessarily genotype-specific, and may be influenced by environmental and developmental fac-

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[*]Corresponding author. Tel. & Fax.: 86-378-3886199; Email: fudeshang@henu.edu.cn
[#]Both are equal contribution to this work.

tors[5]. Unaffected by environmental variables, genetic markers can be used to reliably and accurately identify *O. fragrans* cultivars. The randomly amplified polymorphic DNA (RAPD) technique was employed in initial studies of *O. fragrans* genetic markers[6–9]. Recently, the amplified fragment length polymorphism (AFLP)[10] technique was used in *O. fragrans*[11] and showed many advantages over the technique of RAPD, restriction fragment-length polymorphism (RFLP) and SSR (microsatellites)[12–14].

The objectives of this study were to analyze the genetic relationships and diversity among 46 *O. fragrans* cultivars, and then to use this genetic diversity information to suggest strategies for the germplasm identification, breeding, protection and conservation.

2 Materials and Methods

2.1 Plant materials

We collected mature leaves of 46 *O. fragrans* cultivars from Hubei, Zhejiang, and Guangxi provinces (Table 1) in September 2007. These were dried in Silica gel and stored at – 70 ºC.

2.2 Genomic DNA extraction

DNA was extracted from 0.5 g – 0.6 g of leaf tissue using a modified CTAB method[15,16], then purified with 5 mol/L NaCl, water-saturated ether, and RNase.

2.3 AFLP analysis

We followed the protocol used by Han *et al*[11]. Genomic DNA (250 ng) was digested with *Eco*RI and *Mse*I at 37 ºC for 3 h, and then at 65 ºC for 2.5 h. The digested DNA fragments were ligated to *Mse*I and *Eco*RI adaptors (Table 2) with T4 DNA ligase for 2 h at 25 ºC. The ligated fragments were diluted 10-fold in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA) and used as templates in the 20-cycle[10] preamplification PCR. Ten primer combinations were used for the selective amplification (Table 2). The PCR amplifications were carried out in a PTC-100 Thermal Cycler (MJ Research Inc.).

Amplified products were separated by electrophoresis with 6% denaturing polyacrylamide gels in 1 × TBE buffer. The gels were then stained with 0.1% silver nitrate[17] and air dried overnight before being photographed. Only unambiguous bands were scored. The presence of a band was scored as 1 and the absence of a band was scored as 0.

2.4 Data analysis

Polymorphic AFLP markers were manually scored as binary data with presence as “1” and absence as “0”, and

Table 1. *O. frangrans* cultivars analyzed in this study

Group	Code	Cultivar	Origin
Luteus	HT1	Zaojingui-1	Hubei
	HT2	Zijingui	Hubei
	HT3	Yuanban Jingui-1	Hubei
	HT4	Huangchuan Jingui	Hubei
	HT5	Jinhuataige	Hubei
	HT6	Qiugui-1	Hubei
	ZT1	Qiugui-2	Zhejiang
	ZT2	Jinqiugui‘Jinqiu’	Zhejiang
	ZT3	Wandian Jin	Zhejiang
	ZT4	Congzhong Xiao	Zhejiang
	GT1	Juye Zijingui	Guangxi
	GT2	Yuanye Zijingui	Guangxi
	GT3	Taoye Jingui	Guangxi
	GT4	Zaojingui-2	Guangxi
	GT5	Yuanban Jingui-2	Guangxi
	GT6	Hanxiao	Guangxi
	GT7	Yuanye Jingui	Guangxi
	GT8	Heishan Jingui	Guangxi
	GT9	Heishanzhizhu	Guangxi
	HT7	Zuiyun	Hubei
Albus	HL1	Zigeng Zigui	Hubei
	HL2	Changgengbai	Hubei
	HL3	Yinxing	Hubei
	HL4	Jiangnan Liren	Hubei
	ZL1	Wanyingui	Zhejiang
	ZL2	Yulinglong	Zhejiang
	GL1	Yuanye Ziyingui	Guangxi
	GL2	Chiye Ziyingui	Guangxi
	GL3	Qinyun	Guangxi
	GL4	Yaotiaoshunv	Guangxi
	GL5	Cuizhu	Guangxi
	GL6	Ruichi Yingui	Guangxi
	GL7	Ziyun	Guangxi
Aurantiacus	HA1	Chiye Dangui	Hubei
	HA2	Gecheng Dangui	Hubei
	HA3	Dangui	Hubei
	HA4	Mantiaohong	Hubei
	ZA1	Zhuangyuanhong	Zhejiang
	ZA2	Yingye Dangui	Zhejiang
	ZA3	Hongyan Ningxiang	Zhejiang
	ZA4	Zuijihong	Zhejiang
Asiaticus	HF1	Sijigui-1	Hubei
	ZF1	Sijigui-2	Zhejiang
	ZF2	Tianxiang Taige	Zhejiang
	ZF3	Danzhuang	Zhejiang
	ZF4	Tiannv Sanhua	Zhejiang

only bands showing unambiguous polymorphism were entered into a data matrix. Genetic similarities between pairs of cultivars were determined by calculating simple matching (SM) coefficients[18,19]. Then the similarity matrices were followed by unweighted pair group method with arithmetic mean algorithm (UPGMA) method[20] by using SAHN clustering analysis of NTSYS-pc version 2.1[19].

3 Results

3.1 Results of DNA amplification

The 10 AFLP primer combinations used amplified 436 scorable bands ranging from 0.1 kb to 1.5 kb, of which 269 were polymorphic (Table 2). The primer combination E-AAC/M-CAC yielded the largest number of scorable bands (55). Bands generated by E-AAG/M-CTC had the highest polymorphism ratio (70%). Each *O. fragrans* cultivar had its unique fingerprinting and could be easily distinguished from each other. It fully indicated abundant polymorphism existed between *O. fragrans* cultivars genomes. Part of AFLP bands amplified by primer combination E-AAC/M-CAG was presented in Figure 1.

3.2 Genetic relationship and cluster analysis

The result of cluster analysis is shown on the dendrogram (Figure 2) depicting the pattern of relationships among the studied cultivars. There was obvious difference among different cultivars.

Pair-wise genetic similarity ranged from 0.69 to 0.87. The UPGMA dendrogram revealed two distinct clusters that could be further divided into five groups at GS value of 0.72: (1). The first group consisted of 4 cultivars from Hubei province, including 1 cultivar of Aurantiacus

Table 2. Performance of 10 AFLP primer combinations (primers sequence were also lined out)

Primer combination *Eco*RI/*Mse*I	No. of amplified bands	No. of polymorphic bands	Polymorphism ratio (%)	Primer sequence
AAC/CAG	39	27	69.23	Adaptors
AAC/CAC	55	30	54.55	*Eco*RI 5′ctc gta gac tgc gta cc
AAC/CTC	53	32	60.38	cat ctg acg cat ggt taa 5′
AAG/CTC	40	28	70.00	*Mse*I 5′gac gat gag tcc tga g
AAC/CTG	31	20	64.52	ta ctc agg act cat 5′
ACT/CTC	44	30	68.18	Pre-selective primer
AGC/CTC	43	24	55.81	*Eco*RI (a) 5′gac tgc gta cca att c-a
AGC/CTG	34	18	52.94	*Mse*I (c) 5′gat gag tcc tga gta a-c
AGC/CTA	44	29	65.91	Primers
ACT/CTG	53	31	58.49	*Eco*RI (a) 5′gac tgc gta cca att c-axx*
Total	436	269		*Mse*I (c) 5′gat gag tcc tga gta a–cYY*
Mean	43.6	26.9	61.70	

* XX is null for plus 1 primers; CC, CT, GG, AG, CG for plus 3 primers; YY are null for plus 1 primers; AG, GC, AC, TG for plus 3 primers.

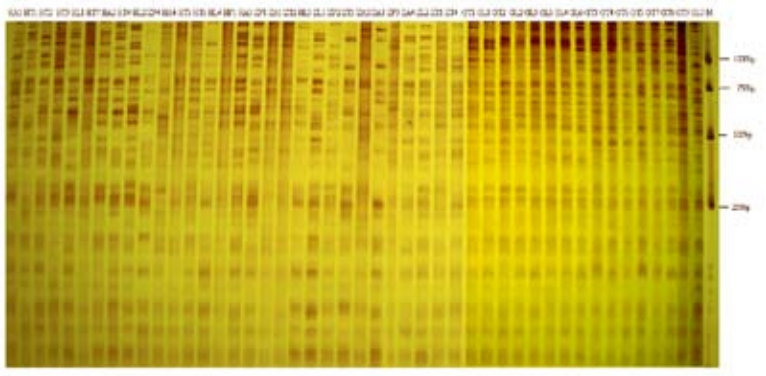

Figure 1. AFLPs generated from genomic DNA of 46 materials using primer combination E-AAC/M-CAG.

Group (HA1) and 3 cultivars of Luteus Group (HT1, HT2, HT4); (2). The second group could be examined more precisely by cutting the dendrogram at GS value of 0.735, the group thus was split to four major sub-groups, leaving HT3 as the first one sub-group and HL1 as the second. There formed 3 minor rami within the third sub-group: the first minor ramus was composed of 3 cultivars (HL2, HL4, HL3) from Hubei province, which belong to Albus Group; the second minor ramus was composed of 2 cultivars (ZF4, ZF3) from Zhejiang province, which belong to Asiaticus Group; the third minor ramus was composed of 5 cultivars from Zhejiang province, including 3 cultivars of Aurantiacus Group (ZA1, ZA2, ZA3) and 2 cultivars of Luteus Group (ZT2, ZT3).The fourth sub-group included 2 cultivars of Aurantiacus Group (HA2, HA4), 2 cultivars of Luteus Group (HT5, ZT1) and 1 cultivar of Albus Group (ZL1); (3).The third group was composed of 2 cultivars of Luteus Group (HT7, HT6) and 3 cultivars of Asiaticus Group (HF1, ZF2, ZF1). (4).The fourth group could be divided into two sub-groups at GS value of 0.72: the first included 2 cultivars of Aurantiacus Group (HA3, ZA4), 1 cultivar of Albus Group (ZL2) and 1 cultivar of Luteus Group (ZT4). They all were collected from Zhejiang province except for HA3, which from Hubei province; the second was exclusively composed of 15 cultivars from Guangxi province, and the 15 cultivars were classified into two sub-groups: one included 5 cultivars of Luteus Group (GT3, GT5, GT4, GT6, GT8); the other included 7 cultivars of Albus Group (GL1, GL2, GL3,GL5,GL4, GL6, GL7) and 3 cultivars of Luteus Group (GT1,GT2,GT9). GL1 and GT2, which were all collected from Guangxi province but belong to different cultivars groups, exhibited the highest genetic similarity of 0.87. (5) The fifth group included only one cultivar (GT7) from Guangxi province, which belongs to Luteus Group.

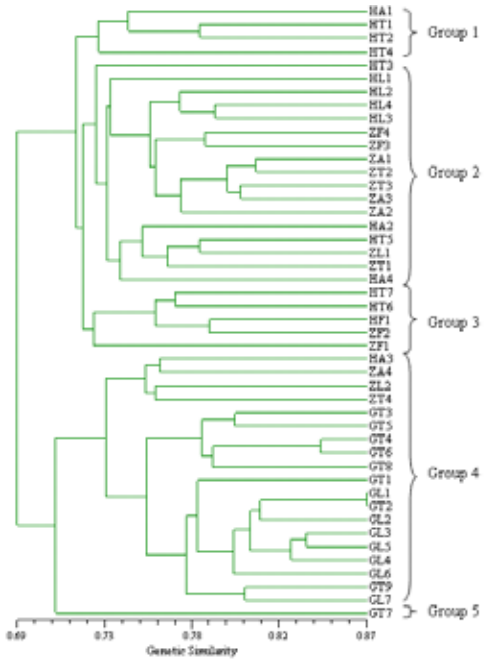

Figure 2. Dendrogram based on simple matching coefficient for 46 materials using ten primer combinations.

4 Discussion

Traditionally, *O. fragrans* cultivars were grouped in either Fragrans Division or Autumn Division according to flowering time. Autumn Fragrans cultivars were further divided into Albus Group, Luteus Group and Aurantiacus Group based on flower color[21–24]. Cluster analysis in present study was not completely in accordance with the traditional classification. The cultivars of the same color could be clustered together at the early stage showed that there were close genetic relationships among cultivars of the same flower color. RAPD and AFLP were used to obtained the similar results from different *O. fragrans* cultivars[6,25]. Zhao *et al* and Liu *et al*, in their analysis of *O. fragrans* cultivars by RAPD[6,25], and Han *et al*, in their AFLP study, obtained the similar results from analysis of different *O. fragrans* cultivars. These similar results suggested that flower color was an advanced criteria of classification which could be used to discriminate efficiently *O. fragrans* germplasm. Cultivars with the same flowering time on the other hand clustered in different groups[25], indicating that flowering time was not a reliable classification criterion. The high natural and artificial selections of this ornamental plant probably has resulted in significant morphological and physiological variations among closely related cultivars, contributing to the discrepancies between the morphology/physiology-based classification and the genetic marker-based groupings. However, these discrepancies may have been exaggerated because the genetic markers used to date possibly do not sufficiently cover regions coding for morphological and physiological traits.

Among the four *O. fragrans* cultivars groups evaluation, the genenic relationship between Luteus Group and Aurantiacus Group was closer than either of them to Albus Group and Asiaticus Group demonstrated by cluster analysis. These data supported the conclusion that Aurantiacus Group cultivars probably originated from bud sports of some Luteus Group cultivars[1,25]. In the light of the laws of evolution and research data, Asiaticus Group

was more primitive than other cultivar groups, while the evolutionary sequence from primitive to advanced in Autumn Division was Albus Group, Luteus Group and Aurantiacus Group[22,27]. Although our conclusion held out this view, there was some difference about the relationship of Asiaticus Group and other cultivar groups. Some researchers obtained the similar conclusion that Asiaticus Group had remote relationship comparing with other three cultivar groups[4,9,11,21,22,25.26]. But, in the dendrogram, the cultivars of Asiaticus Group were clustered with those of other three cultivar groups, which was not in accordance with their conclusion. This difference was probably not typical as a whole because of the limited number of cultivars of Asiaticus Group included in this experiment. So, for further understand the relationship and the evolutive relation of the four cultivar groups, more *O. fragrans* cultivars especially some cultivars of Asiaticus Group were needed.

The geographic origin of *O. fragrans* was correlated with the analysis cluster results at a certain level. Cultivars originating from the same place could be clustered together in the AFLP dendrogram. As was shown in the dendrogram, some cultivars of Hubei were clustered into one group (HA1,HT1, HT2, HT4), some met in one minor group (HL2, HL4, HL3); some cultivars of Zhejiang cultivars were put into one minor group (ZA1, ZA2, ZA3, ZT2, ZT3); all cultivars from Guangxi were clustered into one subgroup with close relationship except GT07. The results confirmed that the *O. fragrans* cultivars from the same place had closer genetic relationships. Cluster analysis indicated that the relationship between Hubei and Zhejiang *O. fragrans* cultivars was nearer and gathered together first, then clustered with Guangxi cultivars, indicating that Guangxi cultivars were distinct from those of Hubei and Zhejiang. Such geographically distinct groups of *O. fragrans* cultivars have been observed in a recent study by Han *et al*[28], and their analysis of the AFLP data also revealed that *O. fragrans* cultivars of the Guilin region formed a distinct subgroup. More studies employing genetic markers are required to map out the geographic distribution of *O. fragrans* cultivar genotypes in China.

This work also established the existence of homonym problems in *O. fragrans* cultivars,the most obvious example is that cultivars with the same name but from different places were clustered into different groups (such as HT1 and GT4, HT3 and GT5, HT6 and ZT1), which suggested that they maybe not the same cultivars. So, we should reexamine the criteria of *O. fragrans* cultivar's classification and establish a rational cultivar classification system.

5 Conclusion

Our study provided valuable information for the identification of *O. fragrans* cultivars and for detecting genetic diversity of the 46 cultivars as well as determining genetic relationships among them. A thorough understanding of the genetic diversity of *O. fragrans* cultivars is critical to future *O. fragrans* cultivars germplasm identification, breeding, protection and conservation. So, further studies may obtain more refined phylogenetic trees of *O. fragrans* cultivars from different geographical areas by combining genetic marker techniques and morphology/physiology-based classification methods.

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