PHYLOGENETIC CLASSIFICATION AS REVEALED BASED ON OPTIMIZE ISSR-PCR SYSTEM IN THE *OSMANTHUS*

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Key words: *Osmanthus*;   ISSR;   systematic classification

Abstract: The species of *Osmanthus* are important gardening plants. But the systematic classification on the molecular level is still not clear. The ISSR (Inter-simple sequence repeat) marker may provide good research evidence for the relationships of the species. In this study, reaction system, based on ISSR analysis in *Osmanthus*., was optimized by gradient PCR examination, and the optimal reaction system was established: 20-100ng template DNA, 2.0mmol/L Mg^{2+}, 0.4umol/L primer, 200umol/L dNTP and 0.5U-1U TaqDNA polymerase. A total of 15 ISSR primers were screened in nine *Osmanthus* Lour. species, of which 4 polymorphic and informative patterns were selected to determine the genetic diversity. ISSR marker analysis suggests that 4 ISSR primers generated a total number of 52 ISSR locus, of which 51 locus were polymorphic, and the percentage polymorphic locus was 98.5%. UPGMA dendrogram showed that these 9 species could be classified into 3 groups: *O.cooperi*, *O.heterophyllus*, *O.×fortunei*; *O.yunnanensis*; *O.fordii*, *O.henryi*, *O.delavayi*, *O.matsumuraanus* and *O.armatus*. The ISSR markers are thus useful in understanding the evolutionary relationships of *Osmanthus*.

INTRODUCTION

Osmanthus. is a member of the family Oleaceae (Scrophulariales, Dicotyledoneae), which includes 31 species and 26 species grow in China. Most of them are widely planted for its important gardening ornamenta. China had successfully obtained the ICAR (international cultivar registration authority) of *Osmanthus* in 2004, which in turn established the important position of China on the research of *Osmanthus*. In the respect of morphologic classification, P.S.Green classified the genus into four groups based on the traits of anthotaxy and flowers: Sect. *Leiolea*, Sect. *Osmanthus*, Sect. *Siphosmanthus* and Sect. *Linocieroides* (Green P.S, 1958). This is the most universally accepted opinion on the classification of *Osmanthus* based on morphologic research at present. Meanwhile, there are research on other aspects such as cytology (Taylor H, 1945), polynology (Xu B.Q et al, 2005), floristic geography (Hao R.M and Zang D.K, 2002) physiology, biochemistry (Zhao X.L et al, 2000) and micromorphology (Ji C.F et al, 2004). As to molecular level, the research on *Osmanthus* mostly focused on its type species *O. fragrans* (Shang F.D et al, 2004; Liu L.C et al, 2004; Hu S.Q et al, 2004; Qiu Y.X et al, 2004). But there is no systematic study on classification and relationships of *Osmanthus* based on molecular methods presently.

The inter-simple sequence repeat (ISSR) PCR using primers based on dinucleotide, tetranucleotide or pentanucleotide repeats has now become in fashion among the researches (Zietkiewicz E et al, 1994). The ISSRs are the regions that lie within the microsatellite repeats and offer great potential to determine intra-genomic and inter-genomic diversity compared to other arbitrary primers, since they reveal variation within unique regions of the genome at several loci simultaneously (Zietkiewicz E et al, 1994). ISSR loci offer several advantages over other molecular markers, such as the

rapid production of a large number of markers, higher reproducibility, Mendel's mode, easily performed at small institutions, no radio-labeling, quickness and the fact that there is no prior need for DNA sequence information from the organism under study. It has since been applied for cultivar identification (Blair M.W et al, 1999), detection gene-flow and introgression (Timmons AM et al, 1996; Allainguillaume J et al 1997), examination genetic diversity and relationships (Shang F.D et al, 2004), studying gene tagging (Ratnaparkhe M.B et al, 1998) and mapping purposes (Kojima T et al, 1998). However, ISSR is a molecular marker based on PCR, so the reaction system is sensitive to many factors. For example, the concentration of template DNA, Mg^{2+}, primer, dNTP and TaqDNA polymerase can all affect the amplification results of ISSR-PCR.

In this work, the effects of different elements on ISSR-PCR products were tested to determine and establish the optimal PCR reaction system for ISSR analysis in *Osmanthus*. And on the basis of the optimal reaction system, the relationships and systematic classification of 9 species in *Osmanthus* were analyzed.

MATERIAL AND METHODS

MATERIAL

PLANT MATERIAL

9 species of *Osmanthus* were analyzed in this study (Table 1). Fresh leaf tissues were collected for DNA extraction.

Table 1 List of plant materials used in this study

Number	species	origin
1	*O.fordii* Hemsl.	Shanghai Guilin Garden
2	*O.henryi* P .S. Green	Kunming Botanic Garden
3	*O. yunnanensis* P. S. Green	Kunming Botanic Garden
4	*O.delavayi* Franch.	Yunnan Jizu Mountain
5	*O.matsumuraanus* Hayata	Hangzhou Botanic garden
6	*O.armatus* Diels	Nanjing Zhongshan Botanic Garden
7	*O. × fortunei* Carr.	Nanjing Zhongshan Botanic Garden
8	*O. heterophyllus* P. S. Green	Shanghai Guilin Garden
9	*O. cooperi* Hemsl.	Hangzhou Botanic garden

METHODS

THE EXTRACTION OF GENOMIC DNA

DNA was extracted from fresh leaves of *Osmanthus* plants following the method of Zou Y P *et al* (Zou Y.P et al, 2001). DNA qualification was tested with 0.8% (W/V) agarose gel electrophoresis. DNA concentration was determined by ultraviolet spectrophotometer (Brim-IOA-0004).

THE OPTIMIZATION OF ISSR-PCR REACTION SYSTEM

Optimized experiments including template concentration (20ng, 40 ng, 60ng, 100ng, 200ng）) $MgCl_2$ concentration (1.5

mmol·L^{-1}, 2.0 mmol·L^{-1}, 2.5 mmol·L^{-1}, 3.0 mmol·L^{-1}, 3.5 mmol·L^{-1}), primer concentration (0.2μmol·L^{-1}, 0.4μmol·L^{-1}, 0.6μmol·L^{-1}, 0.8μmol·L^{-1}, 1.0μmol·L^{-1}), dNTP concentration (100μmol·L^{-1}, 200μmol·L^{-1}, 300μmol·L^{-1}, 400μmol·L^{-1}, 500μmol·L^{-1}) and TaqDNA polymerase concentration (0.5U, 1.0U, 1.5U, 2.0U, 2.5U) were performed to establish proper reaction system.

PCR amplification was performed in the following procedure: after initial denaturation for 5min at 95°, then 35 cycles were performed with 1min at 94°, 1min at 52°, 2min at 72°, and a final extension of 8min at 72°.The PCR products were analysed by electrophoresis using a 1.5% (W/V) agarose gel in 1×TAE buffer (0.04M Tris-acetate, and 0.001M EDTA). Gels were run at 80V for 3 handstained by soaking in a 1 μl/10ml ethidium bromide solution for 15 min. The ISSR banding patterns on gels were visualized using a photo-UV transilluminator system and recorded by digital photography.

PRIMERS SCREENING

Every species selected DNA template randomly from two accessions. Using DDW as negative control, 15 primers (Nanjing Sunshine Biotechnology Co. Ltd) were screened and four primers which could amplify distinctly and repetitively were selected for PCR amplification (Table 2).

DATA ANALYSIS

Each amplification fragment generated by PCR was treated as a unit character and scored as present (1) or absent (2).Genetic distances and genetic identity were calculated and were used to construct a dendrogram by an unweighted pair-group method with arithmetical averages (UPGMA).

RESULTS AND ANALYSIS

THE OPTIMIZATION OF ISSR-PCR REACTION SYSTEM

Using intensity of the locus and number of the smear bands as criterion, the PCR amplified products were analyzed. The results (Fig 1) showed that the concentration of template DNA and TaqDNA polymerase have little influence on the results of this study. On the contrary, the concentration of Mg^{2+}, primers and dNTP have great influence on the PCR amplified results. So the following optimal reaction system was established: 20-100ng template DNA, 2.0mmol/L Mg^{2+}, 0.4umol/L primer, 200umol/L dNTP and 0.5U-1U TaqDNA polymerase.

ANALYSIS OF ISSR POLYMORPISM

Randomly selecting two accessions, 15 primers were screened, of which 4 could generate distinct amplification products (Table 2). The results of amplification can be viewed in fig 2, which showed that 4 primers generated a total number of 52 locus, of which 51 were polymorphic, and on the average, one primer could generate 12.8 polymorphic locus, the percentage polymorphic locus was 98.5%. These results revealed that ISSR markers can reflect the inter-species polymorphism of *Osmanthus*, and can be used for systematic classification study on *Osmanthus*.

Table 2   ISSR primers sequence and amplified results

Primer	Primer Sequence	Nl	Npl	Ppl（%）
ISSR1	5' ACACACACACACACACT 3'	12	12	100.0
ISSR3	5'ACACACACACACACACTT 3'	13	13	100.0
ISSR4	5'ACACACACACACACACAG 3'	11	10	90.9
ISSR9	5' CTCCTCCTCCTCCTCCTC 3'	16	16	100.0
Total		52	51	
Mean		13.0	12.8	98.5

Note：Nl: No. of locus; Nbl: No. of polymorphic locus; Pbl: percentage of polymorphic locus.

ANALYSIS OF SPECIES RELATIONSHIP

On the basis of the opinion that the remoter the genetic distance, the farther the genetic relationships, the ISSR produced locus were analyzed by the TFPGA software, and the genetic distance and genetic identity of 9 species of *Osmanthus* were obtained (Table 3). Further, the dendrogram of 9 species of *Osmanthus* by UPGMA was constructed (Fig 3). The dendrogram showed that these 9 species surveyed could be classified into 3 groups by the 4 ISSR primers: the first group included *O.cooperi*, *O.heterophyllus* and *O.×fortunei*; *O.yunnanensis* alone constituted the second group; and the third group was consisted of *O.fordii*, *O.henryi*, *O.delavayi*, *O.matsumuraanus* and *O.armatus*.

Table 3   Genetic distance and genetic identity based on ISSR amplification

species	1	2	3	4	5	6	7	8	9
1	*****	0.6731	0.5577	0.6346	0.6538	0.6923	0.6538	0.5577	0.6346
2	0.3959	*****	0.5385	0.6538	0.6346	0.6731	0.5577	0.5000	0.5769
3	0.5839	0.6190	*****	0.6923	0.6731	0.5962	0.5962	0.5385	0.5385
4	0.4547	0.4249	0.3677	*****	0.6731	0.7115	0.6346	0.5769	0.5385
5	0.4249	0.4547	0.3959	0.3959	*****	0.6923	0.6538	0.5192	0.5577
6	0.3677	0.3959	0.5173	0.3403	0.3677	*****	0.7308	0.7115	0.6731
7	0.4249	0.5839	0.5173	0.4547	0.4249	0.3137	*****	0.7885	0.7500
8	0.5839	0.6931	0.6190	0.5500	0.6554	0.3403	0.2377	*****	0.7308
9	0.4547	0.5500	0.6190	0.6190	0.5839	0.3959	0.2877	0.3137	*****

Note: Nei's genetic distance (below diagonal) and genetic identity (above diagonal); 1-9:Number of sample.
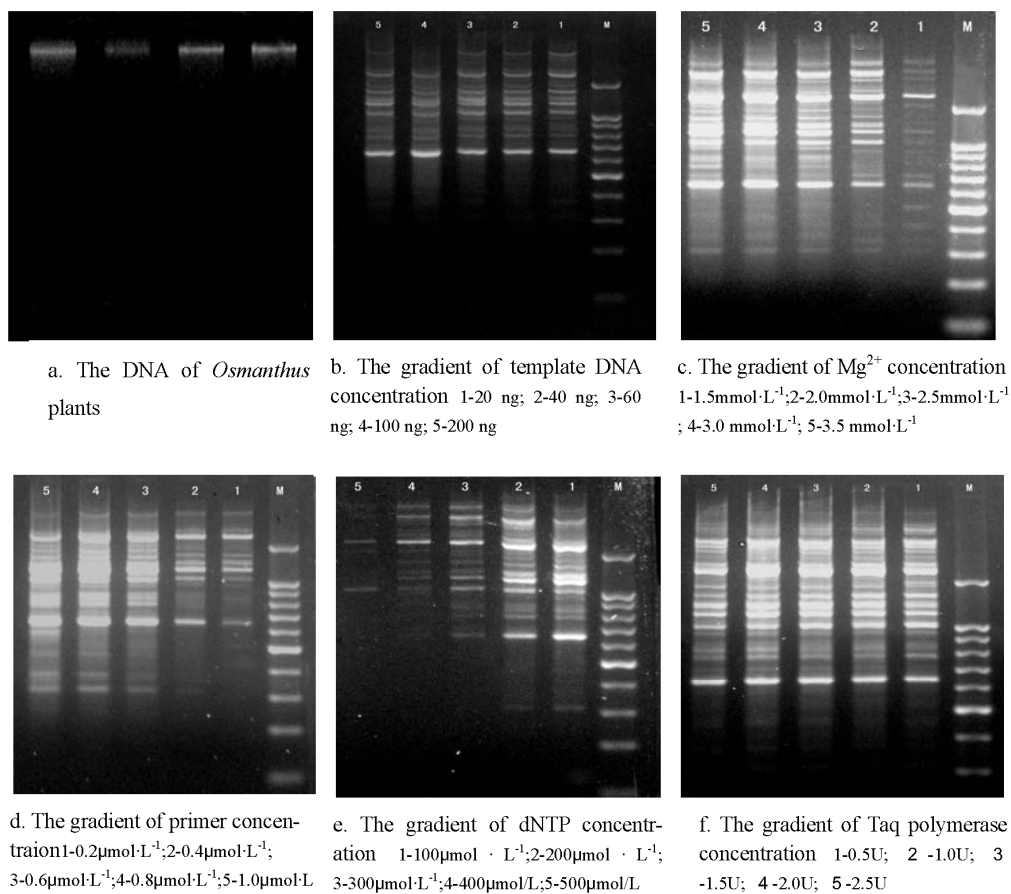
a. The DNA of *Osmanthus* plants

b. The gradient of template DNA concentration 1-20 ng; 2-40 ng; 3-60 ng; 4-100 ng; 5-200 ng

c. The gradient of Mg^{2+} concentration 1-1.5mmol·L^{-1};2-2.0mmol·L^{-1};3-2.5mmol·L^{-1} ; 4-3.0 mmol·L^{-1}; 5-3.5 mmol·L^{-1}

d. The gradient of primer concentraion1-0.2μmol·L^{-1};2-0.4μmol·L^{-1}; 3-0.6μmol·L^{-1};4-0.8μmol·L^{-1};5-1.0μmol·L

e. The gradient of dNTP concentration 1-100μmol · L^{-1};2-200μmol · L^{-1}; 3-300μmol·L^{-1};4-400μmol/L;5-500μmol/L

f. The gradient of Taq polymerase concentration 1-0.5U; 2 -1.0U; 3 -1.5U; 4 -2.0U; 5 -2.5U

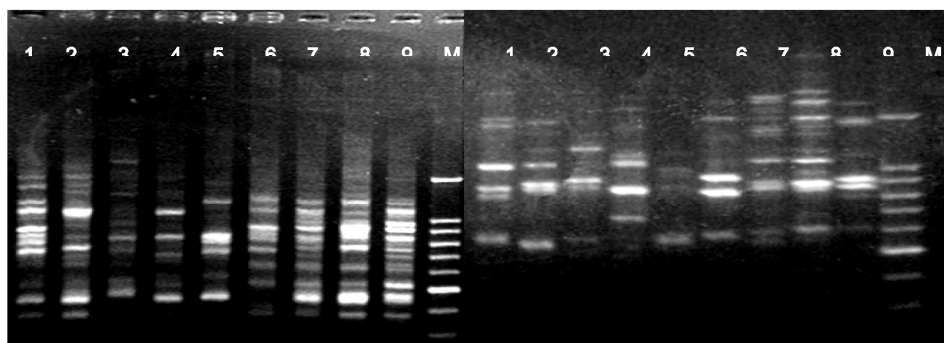Fig 1 optimized results of ISSR-PCR amplification system

Fig 2   ISSR-PCR fingerprints of primer ISSR1(A) and ISSR9(B).   (1-9, Number of sample; M, molecular marker)
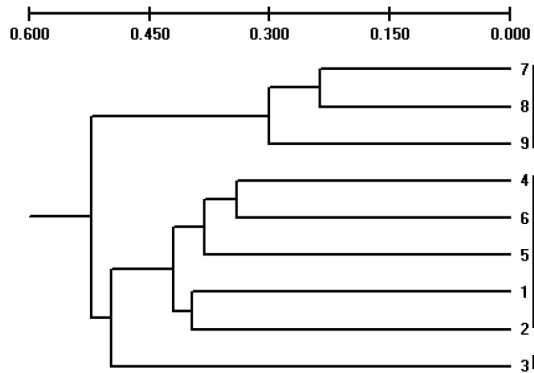
Fig 3   UPGMA dendrograms of 9 Osmanthus. species constructed based on Nei's genetic distance (Population codes are the same as in Table 1.)

DISCUSSION

ISSR technique is subject to the influence of reaction conditions. Different researchers found that many factors such as primer concentration (Bruno W.S, 1993) and dNTP concentration (Zhang H.Q et al, 1999) had great influence on ISSR-PCR amplified results. In this study, Mg^{2+} concentration, primer concentration and dNTP concentration had more influence than TaqDNA concentration and template DNA concentration on the reaction system. Therefore, it is very necessary to optimize its reaction system before ISSR analysis.

Based on the relatively classic classification system put forward by P.S.Green whom used the traits of anthotaxy and flowers, the 9 species surveyed in this study can classified into three groups: *O.delavayi* belonged to Sect.*Siphosmanthus*, *O.matsumuraanus* falled into Sect.*Leiolea*, and the others were all included in Sect.*Osmanthus*. However, the classification results in this study based on the ISSR-PCR was not consistent with Green's. According to the UPGMA dendrogram, the 9 species tested in this study can classified into three groups also. *O.cooperi*, *O.heterophyllus* and *O.×fortunei* were grouped together, this result was accordance with the traditional classification viewpoints (Green P.S, 1958; (Ji C.F et al, 2004). As it was shown in table 3, the genetic identity between *O. heterophyllus* and *O.× fortunei* was the highest (0.7885) which perhaps could provide more foundation for the opinion that *O.× fortunei* was the hybrid of *O. heterophyllus* and *O.fragrans*. In addition, *O.yunnanensis* alone clustered a group, *O.fordii*, *O.henryi*, *O.delavayi*, *O.matsumuraanus* and *O.armatus* grouped together. *O.yunnanensis* belonged to Sect.*Osmanthus* according to the traditional opinion, but here it was alone a group, this was consistent with the result based on ITS, which our lab are proceeding (unpublished). Obviously, though Green's classification viewpoint on *Osmanthus* has extensively accepted yet

(Green P.S, 1958) , his conclusions drawn from herbarium specimens should need integrating molecular biotechnology for further validation.

Presently, the research on *Osmanthus* mostly focused on the morphologic traits, but many species of *Osmanthus* are difficultly distinguished from morphologic traits which block the research work on this genus because of absence of systematic study at the molecular level. As far as the the results in this study are concerned, perhaps, the morphologic evolution is not in concordance with the molecular evolution in *Osmanthus*. So in the future time, advanced molecular biotechnology should be applied for the systematic classification study, and combined with the traditional classification to establish a scientific classification system in *Osmanthus*.

REFERENCES

1.   Green P.S. A monographic revision of *Osmanthus* in Asia and America. Notes from the Royal Botanic Garden Edinburgh, 1958, 22 (5):439-542

2.   Taylor H. Cyto-taxonomy and phylogeny of the Oleaceae. Brittonia, 1945,5:337-367

3.   Xu B.Q, Hao G, Hu X.Y. Pollen morphology of *Osmanthus* (Oleaceae) in China and its systematic significance. Journal of Tropical and Subtropical Botany, 2005, 13 (1): 29-39 (in Chinese)

4.   Hao R.M, Zang D.K. A study on the floristic geography of *Osmanthus*. In:Xiang Q B ed. Sweet Osmanthus-collection on the application to international cultivar registration authority (ICRA) of Osmanthus fragrans cultivars . Jinlin: Jilin Science and Technology Press, 2002. 39-44 (in Chinese)

5.   Zhao X.L, Yao C.H, Wang C.Y. Studies on the isozyme of sweet osmanthus varieties. Journal of Huazhong Agricultural University, 2000, 19 (6):595-599 (in Chinese)

6.   Ji C.F, Xiang Q.B, Xu B.S. Characters of leaf micromorphology of the genus *Osmanthus* (Oleaceae) and its application in systematic taxonomy. Journal of Nanjing Forestry University (Natural Sciences Edition), 2004, 28 (4):54-58 (in Chinese)

7.   Shang F.D, Yin Y.J, Zhang T. The PAPD analysis of 17 *Osmanthus fragrans* cultivars in Henan province. Acta Horticulturae Sinica, 2004, 31(5): 685-687 (in Chinese)

8.   Liu L.C, Xiang Q.B, Liu Y.L. The application of RAPD markers in diversity detection and cultivars identification of *Osmanthus fragrans*. Journal of Nanjing Forestry University (Natural Sciences Edition), 2004, 28, add.:76-82

9.   Hu S.Q, Qiu Y.X, Wu G. H, Xuan Z.C. A ISSR-PCR analyze of the *Osmanthus fragrans* cultivars. Journal of Nanjing Forestry University (Natural Sciences Edition), 2004, 28, add.:71-75 (in Chinese)

10.   Qiu Y.X, Hu S.Q, Chen Y.L, Chen X, Wu G. H. Studies on cultivar classification of *Osmanthus fragrans* by ISSR-PCR analysis. Acta Horticulturae Sinica, 2004, 31(4):529-534 (in Chinese)

11.   Zietkiewicz E, Rafalski A, Labuda D. Genome fingerprinting by simple sequence repeat (SSR)-anchored

polymerase chain reaction amplification. Genomics, 1994, 20:176-183

12.   Blair M.W, Panaud O, Mccouch S R. Inter-simple sequence repeat (ISSR) amplification for analysis of microsatellite motif frequency and fingerpinting in rice (*Oryza sativa* L.). Thear appl Genet, 1999, 98:780-792

13.   Timmons AM, Charters YM, Crawford JW, Burn D, Scott SE, Dubbels SJ, Wilson NJ, Robertson A, O'Brien ET, Squire GR,Wilkinson MJ. Risks from transgenic crops. Nature, 1996, 380: 487

14.   Allainguillaume J, Wilkinson MJ, Clulow SA, Barr SNR. Evidence that genes from the male parent may influence the morphology of potato dihaploids. Theor Appl Genet, 1997, 94: 241-248

15.   Ratnaparkhe M.B, Santra D.K, Tullu A, Muehlbauer F.J, Inheritance of inter-simple-sequence-repeat polymorphisms and linkage with a fusarium wilt resistance gene in chickpea. Thear appl Genet, 1998, 96:348-353

16.   Kojima T, Nagaoka T, Noda K. Genetic linkage map of ISSR and RAPD makers in Einkorn wheat in relation to that of RFLP makers. Thear appl Genet, 1998, 96:37-45

17.   Zou Y.P, Ge S, Wang X.D. The molecular markers in systematic and evolutionary botany. Beijing: Science Press, 2001.16-17 (in Chinese)

18.   Bruno W.S. High output genetic mapping of polyploids using PCR-Generated junkers. Thear appl Genet, 1993, 86:105-112

19.   Zhang H.Q, An L.J, Zu Y.G. Effect on the experimental results of the content of compositions in RAPD experiment of *Pinus koraiensis*. Bulletin of Botanical Research, 1999, 19:183-186 (in Chinese)

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