STUDY ON THE POLYMORPHISM OF ITS IN *OSMANTHUS*

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Abstract In this study, ITS sequence was cloned respectively from 17 species of *Osmanthus* and sequenced. It was found that length of ITS sequence including 5.8S of 17 species ranged from 614bp to 619bp, 5.8S was composed of 163bp. Furthermore, ITS sequence of individuals species took on polymorphism, the similarity coefficient between ITS sequences of every species was 97.90%~100%, the minimum similarity coefficient appeared in *O.reticulatus* (97.90%~99.50%), and the maximum is shown in *O.cooperi* (99.70%~100%). 58 kinds of ITS sequences from 17 species were analyzed and discovered that the polymorphic ITS sequences from 12 species were respectively clustered together, but 3 kinds of ITS sequences from *O.Venosus* were not clustered together, thus apart from *O.Venosus*, any of ITS sequence clustered together from the other 16 species of *Osmanthus* all could stand for the feature of ITS sequence in every species. In addition, in the dendrogram, *O.yunnanensis* and *O.attenuatus* were clustered to be one group, *O.americanus*, *O.matsumuraanus* and *O.delavayi* were clustered to different groups, the other 12 species of were clustered together, which is partly identical with the viewpoint of traditional taxology about *Osmanthus*. Therefore, it might be feasible to analyze the sibship and phylogenetic relationships of *Osmanthus* by ITS.

INTRODUCTION

Osmanthus belong to the Oleaceae, are comprised of about 31 species, and most are important ornamental plants. According to the traits of anthotaxy and flowers, *Osmanthus* were classified into four groups by Green, Sect.*Leiolea*, Sect.*Osmanthus*, Sect.*Siphosmanthus* and Sect.*Linocieroides* (Green 1958), which is one universally accepted opinion about classification of *Osmanthus* based on morphologic research at present. Meanwhile, there are other studies such as cytology (Taylor 1945), palynology (Xu et al 2005), physiology and biochemistry (Zhao et al 2000), and micromorphology (Ji et al 2004). However, there is no systematic study on classification and sibship of *Osmanthus* by molecular methods, studies of *Osmanthus* mostly were focused on the type species *O.fragrans* (Shang et al 2004; Liu et al 2004; Hu et al 2004; Qiu et al 2004), which would lay great foundation on research of *Osmanthus*. Furthermore, Wallander and Albert in 2000 assessed the phylogenetic relationships among 76 species of Oleaceae according to *rps16* and *trnL-F* sequences, and discovered that five genera of Oleinae, such as *Osmanthus*, *Picconia*, *Phillyrea*, *Nestegis* and *Notelaea* manifested close relationships which was consistent with the morphologic and anatomy data (Wallander and Albert 2000). Therefore, the reliable results could be obtained by combination between molecular classification technique and traditional classification index.

Recently, some molecular markers, such as RFLP, AFLP, RAPD, and the like, can provide dependable information about phylogeny of plants, in which analysis of ITS (internal transcribed spacer, ITS) has been widely used to study the phylogenetic relationships among species or genera in plants (Hsiao et al 1995; Lu et al 2000; Liu et al 2000). ITS is located in the interval region of ribosome DNA, and shows highly conservative, rapid evolution speed and short length,

thus the genetic diversity and sibship among genera or species would be studied conveniently with ITS. However, ITS sequence of *Osmanthus* has hardly been reported. In this research, ITS sequences from 17 species of *Osmanthus* were analyzed and their relationships were also discussed on the molecular level in order to provide more data for classification and bipship among species of *Osmanthus*.

MATERIALS AND METHODS

Plant materials

In this article, the fresh leaves of 17 species in *Osmanthus* were collected respectively, quickly dried in the silica gel, and then stored at -80℃ for DNA extraction. The origin of materials and other information were shown in Table 1.

Table 1 Plant materials collected and used in this study

Code	Species	Locality	Voucher	GenBank accession No.	Remark
1	*O.americanus*	Botanic Garden of Harvard University, America	T.R.Dudley 124 (PE)	EF362761	(1)
2	*O.serrulatus*	Emeishan,Sichuan, China	Q.B.Xiang 200111 (NF)	EF199709	(4)
3	*O.reticulatus*	Fanjingshan, Guizhou, China	F.T.Wang 3916 (PE)	EF362765	(4)
4	*O.pubipedicellatus*	Wuhan Botanical Garden, China	H.D.Zeng 21629 (PE)	EF362758	(4)
5	*O.Venosus*	Wuhan Botanical Garden, China	G.H.Yang 58087 (PE)	EF362762	(4)
6	*O.fragrans*	Fanjingshan, Guizhou, Chian	X.Q.Wang 533 (NF)	EF362763	(4)
7	*O.attenuatus*	Wuhan Botanical Garden, China	X.H.Song 1189 (NF)	EF362768	(4)
8	*O.marginatus*	Huangshan, anhui, China	Y.F.Deng 11873(NF)	EF362759	(1)
9	*O.fordii*	Guilin Park, Shanghai, China	Z.Y.Chen 53318 (NF)	EF362764	(4)
10	*O.henryi*	Kunming Botanical Garden, China	G.G.Tang 1265 (NF)	EF362766	(4)
11	*O. yunnanensis*	Kunming Botanical Garden, China	H.Y.Zhou	EF362760	(4)

			10086 (NF)		
12	*O.delavayi*	Jizushan, Yunnan, China	G.G.Tang 1248 (NF)	EF362767	(3)
13	*O.matsumuraanus*	Hangzhou Botanical Garden, China	Q.W.Wang 73641(NF)	EF362770	(1)
14	*O.armatus*	Zhongshan Botanical Garden, Nanjing, China	Y.Chen 3018 (NF)	EF362769	(4)
15	*O.× fortunei*	Zhongshan Botanical Garden, Nanjing, China	Y. Chen 11093(NF)	EF409350	(4)
16	*O.heterophyllus*	Guilin Park, Shanghai, China	K.Ling 943 (NF)	EF362771	(4)
17	*O.cooperi*	Hangzhou Botanical Garden, China	X.Y.He 3058 (NF)	EF362772	(4)

Annotation: NF, PE and KUN represent respectively Herbarium of Nanjing Forestry University in China, Herbarium Beijing Institute of Botany Chinese Academy of Sciences, Herbarium Kunming Institute of Botany Chinese Academy of Sciences. (1), (3) and (4) indicate Sect.*Leiolea*, Sect.*Siphosmanthus*, Sect.*Osmanthu*, respectively.

Methods

DNA extraction

Total genomic DNA was extracted from fresh leaves of *Osmanthus* using CTAB (cetyltriethyl ammonium bromide) with modifications. Yield and purity of genomic DNA was estimated by spectrophotometry at 260nm, and the integrity of genomic DNA was determined by denaturing agarose gel electrophoresis.

PCR amplification

Two pairs of PCR primers were used in this research, the first pair was designed based on the terminal sequences of 18S rDNA and 26S rDNA from *Olea*, *FraxinusL* and *abdiophyllum* which are near to *Osmanthus*, a1: 5′-GAAC(TC)TGCGGAAGGATCAT(TC)G-3′, and a2: 5′-CTGACCTG(GA)GGTCGC(AT)GTCG-3′. The second pair was general primer designed by White et al (1990), b1: 5′-GGAAGTAAAAG TCGTAACAAGG-3′, and b2: 5′-TCCTCCTCCGCTTATTGATATGC-3′.

PCR amplification was performed according to the following procedure: firstly predegenerated for 5min at 95℃, then 28 cycles were carried through as the following, 1min at 94□, 1min at 56℃~57□, 2min at 72□, respectively, and finally ending with 8min at 72□. In addition, PCR products were separated with 1.5% agarose gel electrophoresis.

DNA sequencing

Sequencing of PCR products and clones was performed in BGI Life Tech Co., Ltd. (Huada, Beijing, China) by ABI373A automatic sequencer. The terminal of all ITS sequences was confirmed based on ITS sequence of *O.fragrans* (GenBank accession No. AF135190), furthermore, ITS sequence from every species was sequenced at least three times.

Data analysis

ITS sequences from 17 species of *Osmanthus* were compiled and arranged by Clustal X, and suitably adjusted according to gap. Afterward, ITS sequences were analyzed with PAUP 4.0, and *Olea europaea* was designated as outgroup (GenBank accession No. AJ585193). The MP (Maximum Par-simony) tree was obtained by Heuristic, the boot strap analyses (1000 replications) were performed to test confidence of every branch in dendrogram.

RESULTS AND DISCUSSIONS

Sequencing of ITS sequence

In this experiment, PCR amplification products of ITS sequence from 17 species of *Osmanthus* were directly sequenced. The sequencing of PCR productions was successful and very ideal, the peak pattern is clear, and the background is lower. However, the heterozygous sites were discovered in several positions (Fig. 1), which indicates the polymorphism possibly occurs in ITS sequence of individual species among *Osmanthus*. In order to confirm whether the polymorphism of ITS sequence appears in individual species of *Osmanthus*, 3~15 clones containing ITS sequence from each species were respectively selected, and in all 95 clones from 17 species were selected to be sequenced.

ITS sequence of *Osmanthus*

It was found that length of ITS sequence (including 5.8S) from 17 species of *Osmanthus* ranged from 614bp to 619bp, 5.8S was composed of 163bp. As shown in Table 2, there were 60 kinds of ITS sequences among 95 clones, such as 10 kinds of ITS sequences in *O.fragrans*, 3 kinds of sequences in *O.fordii*. Furthermore, ITS

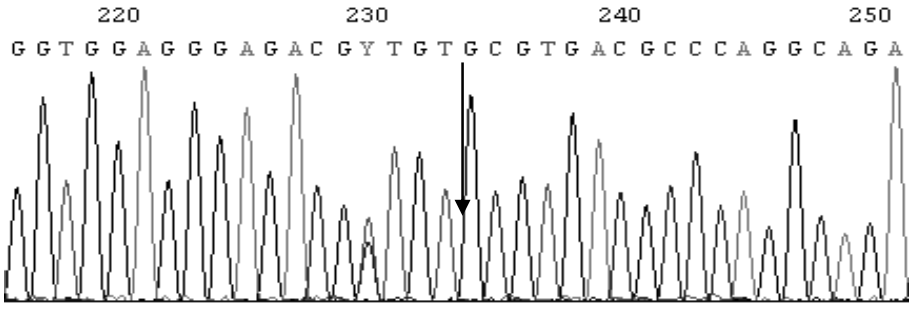

Fig. 1 The sequencing graph of PCR products sequenced directly
The arrow denotes heterozygous loci

Table 2 The similarity coefficient of ITS between clones sequenced of every specie

Code	No. of clones sequenced	No. of different clones	Similarity coefficient
1	15	10	98.5%~100%
2	8	3	98.50%~100%
3	7	3	99.70%~100%
4	5	3	99.20%~99.7%
5	5	3	98.50%~99.3%
6	3	3	99.00%~100%
7	5	4	97.90%~99.5%
8	5	3	98.50%~99.4%
9	4	4	98.40%~98.7%
10	3	3	99.50%~100%
11	5	3	98.70%~99.8%
12	5	4	98.20%~98.9%
13	5	3	99.00%~100%
14	5	3	99.40%~100%
15	5	4	98.70%~100%
16	5	5	98.20%~100%
17	5	3	98.90%~99.4%

Note: 1-17 were number of sample and represent respectively *O.americanus*, *O.serrulatus*, *O.reticulatus*, *O.pubipedicellatus*, *O.Venosus*, *O.fragrans*, *O.attenuatus*, *O.marginatus*, *O.fordii*, *O.henryi*, *O.yunnanensis*, *O.delavayi*, *O.matsumuraanus*, *O.armatus*, *O.× fortunei*, *O. heterophyllus*, *O.cooperi*.

sequence from any one of 17 species all exhibited different, which indicates that polymorphism of ITS generally appears in individual species of *Osmanthus*. The similarity coefficient between different ITS sequences from every species was listed in table 2 and ranged from 97.90% to 100%, the minimum similarity coefficient appeared in *O.reticulatus* (97.90%~99.50%), but *O. cooperi* took on the maximum similarity coefficient (99.70%~100%).

However, what is the reason or mechanism resulting in the polymorphism of ITS sequence in every species of *Osmanthus* need be further studied. Furthermore, the divergence in ITS sequence frequently happened in some population, such as *Tripsacum* and *Zea* (Edward and

Timothy 1996), *Larix* and *Pseudotsuga* (Gernandt and Liston 1999), *Quercus* (Muir et al 2001), *Leucaena* (Hughes et al. 2002), *Armeria* (Feliner et al 2004), and in animals like *Simulium damnosum* (Tang et al 1996), *Meloidogyne* (Hugall et al 1999), and the like. In this study, polymorphism of ITS sequence all existed in 17 species of *Osmanthus*, for example, there were 10 kinds of ITS sequences in 15 clones of *O.fragrans*, 3 kinds of ITS sequences were discovered in 5 clones of *O.yunnanensis*, 4 kinds of ITS sequences were respectively found in 5 clones of *O.henryi* and *O.armatus*. So, the polymorphism of ITS sequence might be an important feature of *Osmanthus*. In this article, ITS sequences from 17 species of *Osmanthus* were firstly studied, thus ITS sequence frequently occurring in each species was registered in GenBank, and the GenBank accession No. were shown in Table 1.

In addition, there are some mutation and deletion sites in ITS sequence of *Osmanthus*, that is to say, the frequence for one base substituted in a certain site is quite high, and even higher than mutation of other sites. For instance, sequencing results of 15 clones from *O.fragrans* indicated that overturn of nucleotide G/A appeared in the 113th site of ITS, sequencing of 8 clones from *O.fordii* made know overturn of nucleotide G/A in the 58th site and nucleotide C/A in 604 th site of ITS, which might make analysis of systematic classification complicated (Donoghue and Baldwin 1993).

Clustering analysis of polymorphic ITS sequence

Because the polymorphism existed generally in ITS sequence of *Osmanthus*, 58 kinds of ITS sequences from 17 species were selected and sorted with CLASTLX, and then analyzed by PAUA 4.0. As shown in Fig. 2, there are 12 species whose polymorphic sequences were clustered all together, which indicates that their similarity is quite high and any one sequence could represent the feature of ITS in every species. In addition, three kinds of ITS sequences from *O.cooperi*, *O.reticulatus*, *O.armatus*, *O.pubipedicellatus* were selected respectively and analyzed, it was found that two sequences from every species were both clustered together apart, and the other was clustered to a far group. However, three kinds of sequences from *O.Venosus* were not clustered together and distributed in farside group. Therefore, apart from *O.Venosus*, any one of sequences clustered together could stand for the feature of ITS sequence to every species.

Otherwise, we could found in Fig. 2, *O.yunnanensis* and *O.attenuatus* were clustered to be one group, *O.americanus*, *O.matsumuraanus* and *O.delavayi* with more close relationship were clustered to different groups, and the other 12 species of were clustered together, which shown partly identical with viewpoint of traditional taxology, for example, *O.yunnanensis* and *O.attenuatus* in Sect.*Osmanthus* were clustered together, and the other species in Sect.*Osmanthus* also exhibited close sibship. Although *O.americanus* and *O.matsumuraanus* in Sect.*Leiolea* were

not clustered together, exhibited highly close sibship. Therefore, it is feasible to analyze sibship and phylogenetic relationships of *Osmanthus* with ITS sequence, simultaneously, viewpoint and theory of traditional taxology should properly be revised by combining with the evidence from the molecular level.

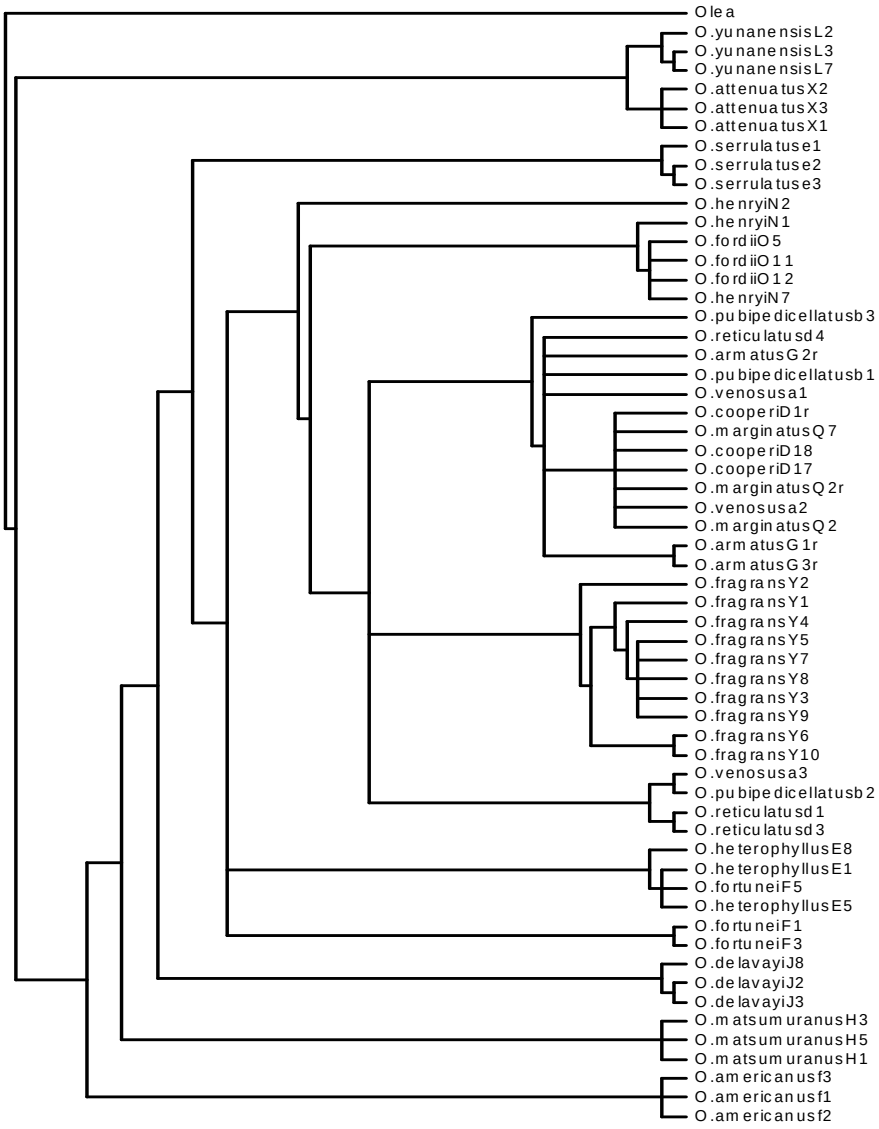

Fig. 2 The MP tree of polymorphic ITS sequences from 17 species of *Osmanthus*

The ITS sequence was composed of ITS-1, ITS-2 and 5.8S, consistency index (*CI*) and retention index (*RI*)

of MP tree were respectively 0.7231, 0.9020, and the outgroup was *Olea europaea*.

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