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STUDIES ON ITS SEQUENCES AND SYSTEMATIC CLASSIFICATION OF *OSMANTHUS*

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Abstract

In this article, ITS sequences of 20 species in *Osmanthus* were cloned and researched. It was found that length of ITS sequences ranged from 614bp to 619bp, in which ITS-1 is obviously longer than ITS-2, 5.8S of 20 species is composed of 163bp, and the percentage of G+C in ITS sequence of 20 species is 54.24%-64.56%. Alignment between ITS sequences from 20 species of *Osmanthus* was performed, and discovered that the similarity coefficient between 20 species is 86.1%-99.7%, the minimum exists between *O.suavi* and *O.attenuatus*, the similarity coefficient between *O.urceolatus* and *O.cooperi* is the maximum. Furthermore, there are 198 mutation sites and 97 information sites in ITS sequences, respectively 31.53% and 15.45%, and the content of information sites in ITS-2 is more than that in ITS-1. In addition, three MP trees were respectively constructed based on ITS sequences, ITS-1 sequences and ITS-2 sequences, 20 species of *Osmanthus* were divided into three groups, *O.americanus* belonged to the first group, *O.yunnanensis* and *O.attenuatus* were clustered one group, and the other 17 species belonged to the third group, which is partly consistent with the viewpoint of traditional classification about *Osmanthus*.

Introduction

The genus *Osmanthus* belongs to Oleaceae. Dicotyledonea, the species are famous ornamentals and mainly distributed in China. At present, the classification basis of *Osmanthus* was built up by Green. There are about 31 species of *Osmanthus* divided into four groups, Sect.*Leiolea,* Sect.*Osmanthus*, Sect.*Siphosmanthus* and Sect.*Linocieroides* according to the flower and inflorescences of *Osmanthus* (Green, 1958). However, experiment materials used by Green were almost dried specimen, and there is little information directly from the fresh material, thus the systematic classification of *Osmanthus* from Green was doubted by many scholars. Furthermore, along with new species of *Osmanthus* emerge in endlessly (Zhang, 1982; Bai, 1983; Song, 1984; Lu, 1989), identification and differentiation of some species are very difficult. Therefore, the phenomenon of synonym and homonym in the *Osmanthus* usually appears, which cause great confusion to the classification of *Osmanthus*. Shang (1985), Wu & Peter (1992) did complete revision of *Osmanthus*, but there is still some bifurcation in naming and classification of several species. In 2004, names of *Osmanthus* were identified and coordinated by Xiang *et al*., and discovered that there are 31 species and 5 varieties. In addition, there are little differences among species of *Osmanthus* and the same species usually express different shape and traits when grown in different environment, which also result in great trouble to the classification of *Osmanthus* (Shang *et al.,* 2002). For instance, it was found that the classification according to shape of pollen existed some divergences against the traditional one (Xu *et al.*, 2005). Other studies also indicated that classification of *Osmanthus* only based on one or just few traits usually has many problems (Ji, 2004). It is well known that DNA molecular marker could clearly reflect on

certain differences in genome of individuals or in population. Presently, there are already many kinds of DNA molecular markers, in which, ITS (internal transcribed spacer) is frequently and extensively applied in systemic classification of plants.

ITS sequence is made up of ITS-1 and ITS-2, which are respectively genetic area between ribosome 18S and 5.8S, ribosome 5.8S and 26S (Tian & Li, 2002; Wang & Liu, 2004). The nucleotide sequences in ITS sequence take on high variability, which can provide rich information for systematic classification, and then conveniently allow people to study genetic diversity and sibship among population or species. Recently, ITS is generally used in studies about systematic evolution and sibship of plant population or species (Chanderbali *et al.,* 2001; Roalson & Friar, 2004), such as the molecular phylogenesis and chromosome evolution of *Hypochaeris* Asteraceae (Cerbah *et al.,* 1998), the evolution relationship of *Actinodaphne* Lauraceae (Li *et al*., 2006), research on sibship of species in *Caragana* Leguminosae (Hou *et al.,* 2006) and classification of *Angelica* Umbelliferae (Xue *et al.,* 2007) and so on, which all proved that it is feasible to study the systematic evolution and sibship of species in dicotyledon by ITS sequence. Until now, it has not reported that ITS sequence was applied in the systematic evolution and sibship of *Osmanthus*. In this article, ITS was used to study and discuss the sibship among species of *Osmanthus* in order to provide more information for classification of *Osmanthus* on molecule level, at the same time to supply theoretical foundation for protection of germplasm resources, cross-breeding and exploitation of *Osmanthus.*

Materials and Methods

Materials: In this article, the experimental materials were fresh leaves of 20 species in *Osmanthus*, which were quickly dried in silica gel and then stored at -80ºC for DNA extraction. Origin of materials and other information were clearly listed in Table 1.

DNA extraction: Total genomic DNA was isolated from leaves of *Osmanthus* by CTAB (cetyltriethyl ammonium bromide) extraction procedure as described previously (Ausubel *et al*., 1987) 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 study, one pair was designed according to terminal sequences of rDNA 18S and 26S from *Olea*, Fraxinus L., and *Abdiophyllum* which are near to *Osmanthus*, ITS1: 5′-GAAC (TC) TGCGGAAGGATCAT (TC) G-3′, and ITS2: 5′-CTGACCTG (GA) GGTCG C(AT) GTCG-3′. The other was the general primer designed by White *et al.,* (1990), ITS3: 5′-GGAAGTAAAA GTCGTAACAAGG-3′, and ITS4: 5′-TCCTCCTCCGCTTATTGATATGC-3′.

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

DNA sequencing: Sequencing of PCR products and clones was performed in BGI Life Tech Co., Ltd. (Huada, Beijing, China) using the ABI373A automatic sequencer. The terminal of all ITS sequences was confirmed according to ITS sequence of *Osmanthus fragrans* (GenBank accession number is AF135190), furthermore, ITS sequences from every species were sequenced at least three times.

Table 1. Plant materials used in this study.

Code	Species	Locality	Voucher	Gen bank 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.Z.Chen 53318 (KUN)	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 10086 (NF)	EF362760	(4)
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)
18.	*O. urceolatus*	Wuhan Botanical Garden, China	G.G. Tang 607 (NF)	EU009481	(4)
19.	*O.racemosus*	Fanjingshan, uizhou, China	X.H. Song 1318 (NF)	EU643798	(1)
20.	*O. suavis*	Hillier Botanical Garden, England	S.E. Liu 15055(PE)	EU009482	(3)

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*, separatively.

Data analysis: ITS sequences from 20 species of *Osmanthus* were compiled and arranged by CLASTLX, and suitably adjusted according to gap. Afterward, ITS sequences were analyzed with PAUP 4.0, and the *Olea europaea* was designated as outgroup. The MP (Maximum Par-simony) tree was obtained by Heuristic, the boot strap analyses (1000 replications) were performed to test the confidence of every branch in phylogenetic tree.

Results

Length and composition of ITS sequence: The ITS sequences of *Osmanthus* were obtained by PCR using ITS1/ITS2 primer or ITS3/ITS4 primer respectively, registered into GenBank, and the GenBank accession numbers are listed in Table 1. As shown in Table 2, the length of ITS sequences including 5.8S (163bp) range from 614bp to 619bp, ITS-1 and ITS-2 is composed of 236bp-239bp or 214bp-217bp, respectively and ITS-1 is obviously longer than ITS-2. In addition, the percentage of G+C in ITS sequence of 20 species is 54.24%-64.56%, and that in ITS-1 is slightly higher than in ITS-2, 54.24%-64.98% or 53.98%-61.20%, separately (Table 2).

The *Olea europaea* was regarded as outgroup, its ITS sequence (including 5.8S) consists of 611bp, ITS-1, ITS-2 and 5.8S is respectively 245bp, 206bp or 163bp, and its GenBank accession number is AJ585193. Alignment between ITS sequences from 20 species of *Osmanthus* and *Olea europaea* is shown in Fig. 1, it was found that 5.8S sequence among 20 species of is same and exhibits highly identical with that of *Olea europaea*. To be further analyzed and discovered that the similarity coefficient among 20 species ranges from 86.1% to 99.7% (Table 3), the minimum is 86.1% and exists between *O.suavi* and *O. attenuatus*, the similarity coefficient between *O. attenuatus* and *O. fragrans*, *O. yunnanensis* and *O. matsumuraanus*, *O. yunnanensis* and *O. racemosus* is also very low (87.1%). However, the similarity coefficient is the maximum between *O. urceolatus* and *O. cooperi* (99.7%), very high between *O. urceolatus* and *O. armatus*, *O. urceolatus* and *O. pubipedicellatus*, *O. urceolatus* and *O. venosus*, *O. urceolatus* and *O. marginatus* (99.4%), and the same high similarity coefficient (99.4%) also appears between *O. cooperi* and *O. armatus*, *O. cooperi* and *O. fordii*, *O. cooperi* and *O. pubipedicellatus*.

Information sites in ITS sequence: The number and percentage of information sites and mutation sites in ITS sequence of *Osmanthus* are shown in Table 4, in ITS sequence (including 5.8S) from 20 species of *Osmanthus*, there are 198 mutation sites (31.53%) and 97 information sites (15.45%), in which 12 mutation sites (7.36%) and 3 information sites (1.84%) exist in 5.8 S. Furthermore, there are 94 mutation sites (38.37%) and 47 information sites (19.18%) in ITS-1, and 92 mutation sites (41.82%) and 47 information sites (21.36%) in ITS-2. In a word, there is a few differences in ITS-1 and ITS-2, and the content of information in ITS-2 is high than that in ITS-1.

Otherwise, the hotspot of mutation and deletion sites was found in ITS sequence of *Osmanthus*, such as, the deletion of A nucleotide in the 125th site which has great effect on the length change of ITS-1, the mutation of G/C nucleotide in the 205th site in which the G/C nucleotide is frequently overturned and the similar frequency of conversion in this site was found in all of experiment samples sequenced.

Clustering analysis of ITS: In this study, the ITS sequences were firstly arranged with CLASTLX, and then the systematic classification was analyzed by PAUA 4.0 using Maximum Par-simony, three MP phylogenetic trees were constructed based on ITS sequence, ITS-1 sequence and ITS-2 sequence from 20 species of *Osmanthus*, respectively (Figs. 2-4).

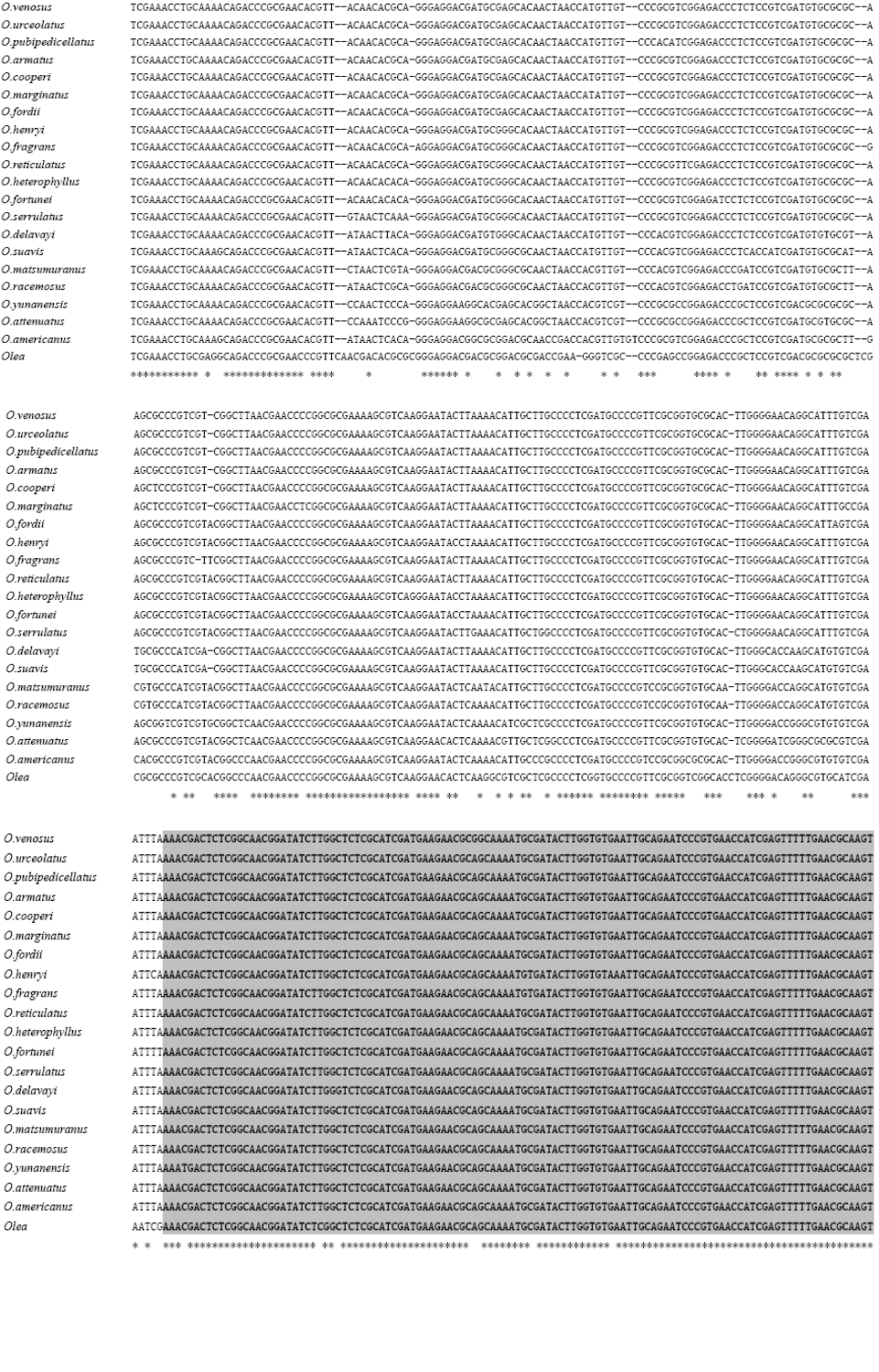
Table 2. Length and G+C content of ITS-1, ITS-2 and ITS.

Species	ITS-1		ITS-2		ITS (including 5.8 S)	
	Length	G+C (%)	Length	G+C (%)	Length	G+C (%)
O. americanus	239	64.02	217	63.13	619	61.07
O. serrulatus	237	57.38	217	56.68	617	55.92
O. reticulatus	237	56.54	216	58.33	616	56.17
O. pubipedicellatus	236	56.36	217	56.68	616	55. 52
O. venosus	236	57.20	217	56.68	616	56.01
O. fragrans	236	56.78	217	55.76	616	55.19
O. attenuatus	237	64.56	216	63.43	616	61.20
O. marginatus	236	56.78	217	55.76	616	55.36
O. fordii	237	56.54	217	55.76	617	55.27
O. henryi	237	57.81	217	55.30	617	55.27
O. yunnanensis	237	63.71	214	61.21	614	60.10
O. delavayi	236	54.24	216	54.63	615	53.98
O. matsumuraanus	237	57.38	217	58.99	617	56.89
O. armatus	236	57.20	217	55.76	616	55.52
O. × *fortunei*	237	56.54	217	57.60	617	55.92
O. heterophyllus	237	57.38	216	55.09	616	55.36
O. cooperi	236	56.78	217	56.22	616	55.52
O. urceolatus	236	57.20	217	56.68	616	55.84
O. racemosus	237	56.96	217	58.06	617	55.56
O. suavis	236	55.51	216	55.56	615	54.80

Table 4. Number and percentage of variable site and informative site in ITS of *Osmanthus*.

	ITS-1		ITS-2		5.8 S		ITS (including 5.8 S)	
	Number	%	Number	%	No.	%	Number	%
Variable site	94	38.38	92	41.82	12	7.36	198	31.53
Informative site	47	19.18	47	21.36	3	1.84	97	15.45

As shown in Fig. 2, Fig. 3 and Fig. 4, the topologic structure of three MP trees are nearly same, especially the MP trees based on ITS sequences (including 5.8S) and ITS-1 sequences are much more similar in the topologic structure of MP tree, and even the branch in MP tree is not obvious, the similarity might reflect on the coevulation relationship between ITS-1 and ITS-2 sequences in some degree. Furthermore, although the content of information in ITS-1 and ITS-2 of 20 species differs, 5.8S provides some information sites, too. Therefore, the clustering results according to ITS sequences would completely exhibit the sibship and systematic classification among species of *Osmanthus*. As shown in Fig. 4, these 20 species of *Osmanthus* were divided into three groups, *O. americanus* belonged to the first group, *O. yunnanensis* and *O. attenuatus* were clustered one group, the other 17 species were clustered to the third group.


O.venosus
O.urceolatus
O.pubipedicellatus
O.armatus
O.cooperi
O.marginatus
O.fordii
O.henryi
O.fragrans
O.reticulatus
O.heterophyllus
O.fortunei
O.serrulatus
O.delavayi
O.suavis
O.matsumuranus
O.racemosus
O.yunanensis
O.attenuatus
O.americanus
Olea

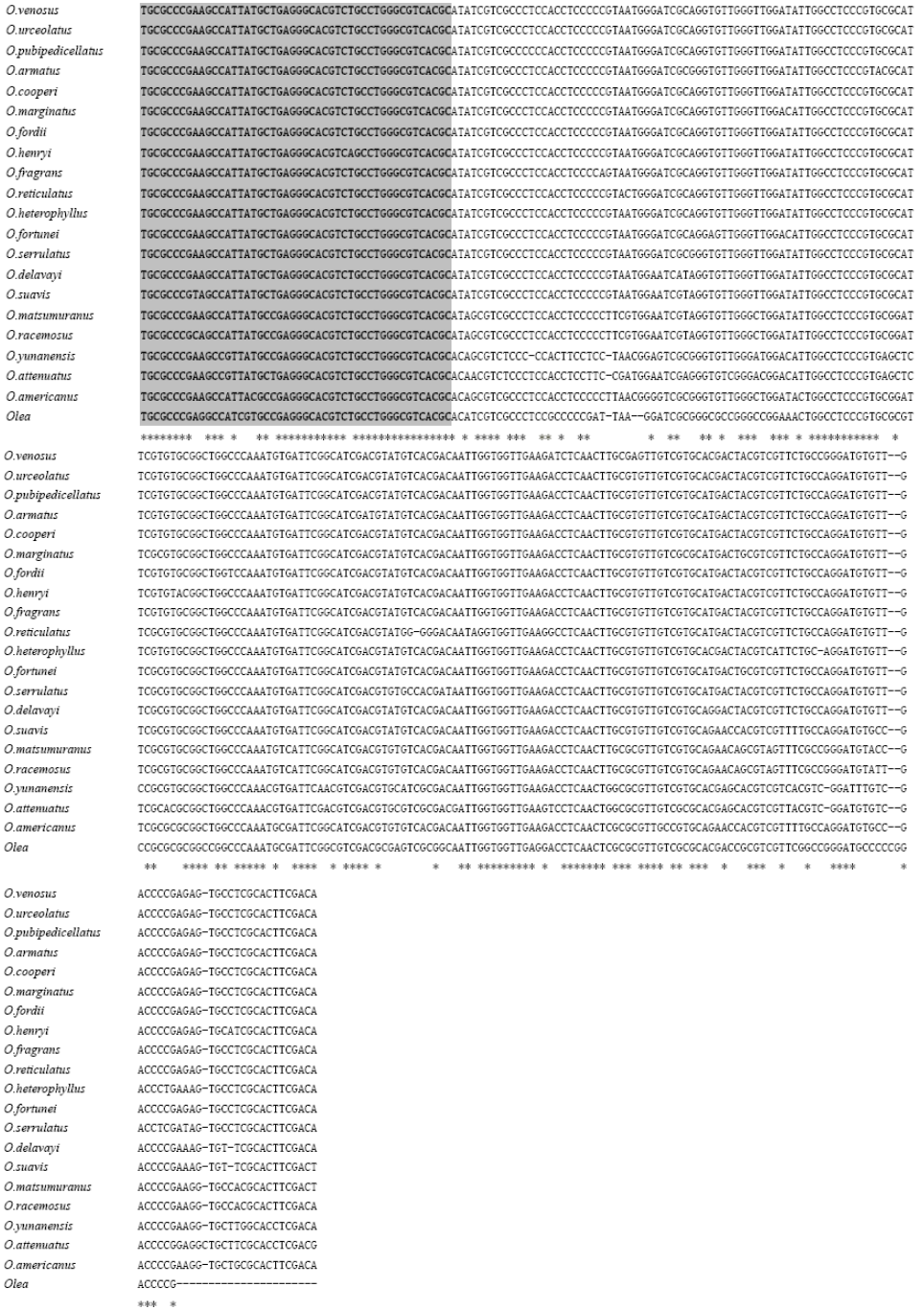


Fig. 1. Alignment between ITS sequences from 20 species of *Osmanthus* and *Olea europaea*. The asterisk (*) indicates the same base, horizontal line (-) represents gap, and the ITS-1 sequence and ITS-2 sequence located respectively front and back of 5.8 S sequence which was shown gray color.

Table 3. The similarity coefficient of ITS sequences between 20 species of *Osmanthus*.

Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.	***																			
2.	90.4	***																		
3.	89.4	96.6	***																	
4.	90.1	96.8	97.7	***																
5.	90.1	96.4	97.4	98.7	***															
6.	90.1	96.8	97.7	98.4	98.1	***														
7.	87.0	87.3	87.3	87.5	87.8	87.1	***													
8.	89.6	96.3	97.2	98.2	97.9	97.6	87.6	***												
9.	90.1	97.1	98.1	99.0	98.7	98.7	88.0	98.2	***											
10.	89.6	96.4	97.4	98.1	97.7	98.4	87.0	97.2	98.4	***										
11.	88.4	87.6	87.4	88.1	88.2	87.6	93.3	87.7	88.1	87.3	***									
12.	90.1	95.3	95.1	95.8	95.3	95.4	86.6	95.0	95.8	95.3	86.7	***								
13.	92.1	91.4	91.1	91.6	91.6	91.2	86.3	91.1	91.6	90.9	87.1	92.4	***							
14.	90.4	97.1	97.7	99.0	98.7	98.4	87.8	98.2	99.0	98.1	88.2	95.4	91.2	***						
15.	90.0	96.9	97.6	97.9	97.6	97.9	87.5	98.1	98.2	97.9	87.8	95.6	91.4	97.9	***					
16.	90.3	96.9	97.4	98.0	98.0	98.0	87.5	97.2	98.4	98.1	87.7	95.9	91.4	98.0	98.2	***				
17.	90.4	97.1	98.0	99.4	99.0	98.7	87.8	98.9	99.4	98.4	88.2	95.8	91.6	99.4	98.2	98.4	***			
18.	90.6	97.1	98.0	99.4	99.4	98.7	88.1	98.5	99.4	98.4	88.6	95.9	91.7	99.4	98.2	98.7	99.7	***		
19.	92.1	91.7	91.7	92.2	92.2	91.9	86.2	91.7	92.2	91.6	87.1	93.0	98.7	91.9	92.1	92.0	92.2	92.4	***	
20.	91.4	94.1	94.0	94.6	94.1	94.3	86.1	93.8	94.6	94.1	86.4	97.2	93.5	94.3	94.5	94.8	94.6	94.8	93.3	***

Note: 1-20 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*, *O. urceolatus*, *O. racemosus*, *O. suavis*.

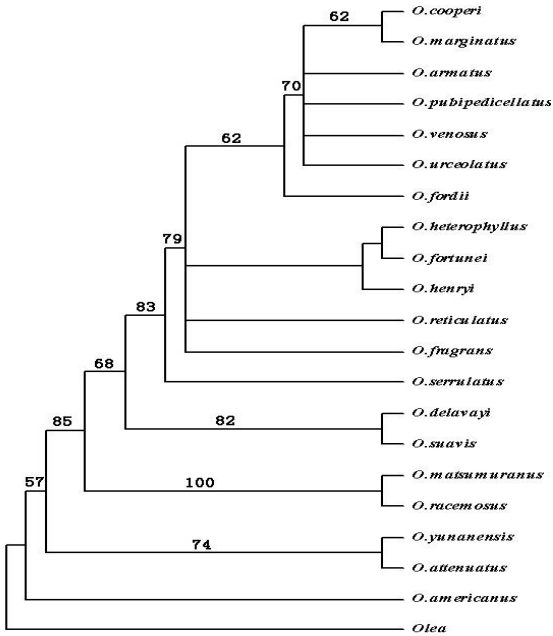


Fig. 2. The MP tree based on ITS-1 sequences from 20 species of *Osmanthus*.
The tree length =144 steps, consistency index (*CI*) = 0.7639, retention index (*RI*) = 0.7571, and the out group was *Olea europaea*.

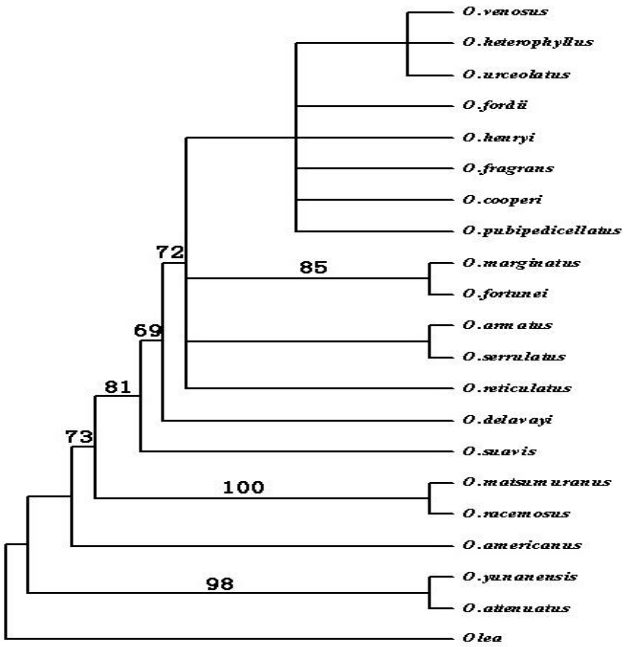


Fig. 3. The MP tree based on ITS-2 sequences for 20 species of *Osmanthus*.
The tree length =151 steps, consistency index (*CI*) = 0.7748, retention index (*RI*) = 0.7531, and the outgroup was *Olea europaea*.

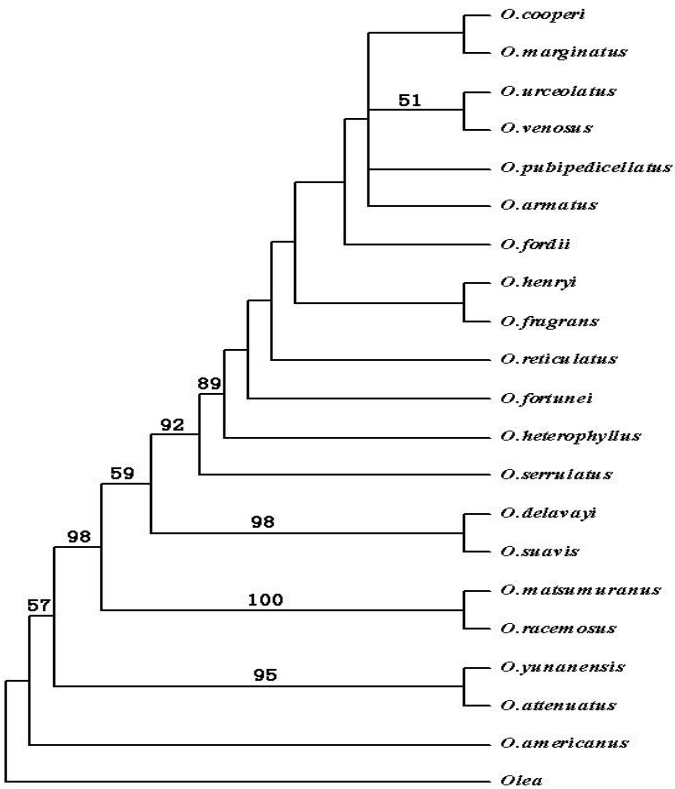


Fig. 4. The MP tree based on ITS sequences from 20 species of *Osmanthus*.
The ITS sequence was composed of ITS-1, ITS-2 and 5.8 S, respectively, tree length =219 steps, consistency index (*CI*) = 0.7586, retention index (*RI*) = 0.7298, and the outgroup was *Olea europaea*.

Discussions

ITS sequences of *Osmanthus*: The length of ITS-1 and ITS-2 is highly conserved, and quite same in one population. It was discovered, ITS-1 sequences of Angiosperm range generally from 187bp to 298bp, ITS-2 sequence is 187bp-252bp, and the length of 5.8S hardly changes, 163 or 164bp (Baldwin *et al.,* 1995). In this research, 5.8S from 20 species of *Osmanthus* is composed of 163bp, ITS-1 and ITS-2 is composed of 236bp-239bp or 214bp-217bp, respectively and the length of ITS sequences range from 614bp to 619bp, which is consistent with the other studies, and also indicates that *Osmanthus* is the natural taxon in the molecular level. Furthermore, the length of ITS-1, ITS-2 and ITS from *O. americanus* in America is 239bp, 217bp, 619bp, respectively and these three sequences are the longest than those from other species of *Osmanthus*, which shows that the species in America has many differences from that in Asia. In addition, total content of G+C in ITS sequence of Angiosperm varies greatly from 50% in some groups to 75% in paddy rice (Nickrent & Starr, 1994). In this research, the content of G+C in ITS sequence is 53.98%-61.20%, that in *O.delavayi* is the lowest, the highest appears in *O.*

attenuatus or *O. americanus*. Therefore, the great difference of G+C content among species of *Osmanthus* indicates that it is feasible to analyze systematic classification of plants by ITS sequence.

Usually, ITS sequence has high variability and could supply rich systematic information. The divergence value of ITS sequences in majority of Angiosperm is 1.2%~10.2% among species, and 9.6%-28.8% among genus, which is appropriate to the study of systematic classification (Baldwin *et al.,* 1995). Moreover, it was found that higher mutation and percentage of information sites in ITS sequence would well support the system reconstruction in population when used to study the relationship among genus or species (Baum *et al.,* 1998; Schwarzbach & Ricklefs, 2000). In this research, in ITS sequence (including 5.8S) of *Osmanthus*, there are 198 mutation sites, 97 information sites, the percentage is respectively 31.53% and 15.45% and the content of information in ITS-2 is more than in ITS-1. Furthermore, the mutation and deletion sites are discovered in ITS sequence of *Osmanthus*, which have not been reported in other plants until now, but the insertion or deletion of nucleotide in ITS sequence might make analysis of systematic classification more complicated (Donoghue & Baldwin, 1993).

Systematic classification of *Osmanthus*: It was found in large number of studies that ITS sequence takes on higher variation speed, can provide rich information sites and especially is suitable for study on systematic classification of plant among species (Baum *et al.,* 1998; Stanford *et al.,* 2000), for example, ITS sequence had an important effect on research about relationship of species in *Rhododendron*, *Metagentiana* (Excoffier, 1993). In this research, the clustering result based on ITS sequences is partly consistent with the viewpoint of traditional taxonomy, for example, *O. matsumuraanus* and *O. racemosus* in Sect. *Spiraea* were clustered in the same branch, *O.delavayi* and *O. suavis* which both belong to Sect. *Siphosmanthus* were clustered together, *O. heterophyllus* and *O. fortunei* in Sect. *Osmanthus* also show very near sibship. However, *O. americanus* in Sect. *Spiraea* was clustered alone, indicating there is obvious difference in the genetic composition between *O.americanus* and *Osmanthus* distributed in China. *O. marginatus* in Sect. *Spiraea* and *O. cooperi* in Sect. *Osmanthus* were clustered together and 14 species in Sect. *Osmanthus* were not clustered the close branch completely, which is inconsistent with the viewpoint of traditional taxonomy. In addition, the sibship among some species of *Osmanthus* was not supported by this research, such as, *O. marginatus* and *O.matsumuraanus*, *O. racemosus*, *O. yunnanensis* and *O. fragrans*, *O. serrulatus*, *O. fortunei* and *O. fragrans*, *O. heterophyllus*, *O. reticulatus* and *O. serrulatus*, *O. cooperi* and *O. fragrans* and so on (Green, 1958).

In brief, the clustering result based on ITS sequences of *Osmanthus* is partially different from the viewpoint of traditional taxonomy, however supports in other aspects, which indicates that the evolution in plant morphology and molecular level are perhaps not synchronous. Otherwise, although the classical viewpoint of *Osmanthus* from Green (1958) is widely accepted in the present time, but it was based on the dried herbarium, therefore, the classification method needs further be confirmed by technology of molecular biology and molecular evidence. In view of the fact that there is a few studies in sibship and systematic classification of *Osmanthus* on the molecular level, and even has been hardly reported through the world, accordingly, the results in this research with differences from the traditional classification need further be validated.

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