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Evaluation of ITS2 for intraspecific identification of *Paeonia lactiflora* cultivars

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ABSTRACT

Herbaceous peony (*Paeonia lactiflora* Pall.) is an important ornamental and medicinal plant. DNA barcodes can reveal species identity via the nucleotide diversity of short DNA segments. In this study, two main candidate DNA barcodes (ITS2 and *psbA-trnH*) were tested to identify twenty-one cutting cultivars of *P. lactiflora* and their wild species. The efficacy of the candidate DNA barcodes was assessed by PCR amplification, sequence quality, sequence diversity, rate of correct identification, and phylogenetic analysis. ITS2 was easy to be amplified and sequenced among the samples. The identification by Blastn and phylogenetic analysis was 95.4% and 63.6%, respectively. For *psbA-trnH*, the presence of poly A-T led to sequencing failure which limited its use as DNA barcode candidate. Moreover, the authentic efficiency of *psbA-trnH* was lower than ITS2. The results showed that ITS2 is suitable as a candidate DNA barcode for the intraspecific identification of *P. lactiflora* cultivars.

1. Introduction

Herbaceous peony (*Paeonia lactiflora*.Pall) belongs to the Paeoniaceae family and is widely distributed in temperate Eurasia as a perennial herbaceous plant [1]. With colorful flowers and wound-healing roots, it is welcomed as a traditional flower, both potted and as cut flowers, and enjoys the title of 'The minister of flowers' in China [2]. The germplasm resource for *P. lactiflora* has over 600 cultivars worldwide [3], and has undergone great changes since being introduced to Europe and America in the 19th century [4]. The modern cultivars used for commercial cut flowers are mostly derived from *P. lactiflora* [5]. *P. lactiflora* will be important in future cut peony breeding due to its wide environmental adaption, strong resistance, and variations that emerge from the seed progenies [6]. Therefore, suitable molecular markers are needed to identify, assess, conserve, and use the germplasm of *P. lactilflora*.

DNA barcode is an efficient method for species-level identification through one or more standard loci that are amplified with universal primers. Barcoding plays an important role in cataloguing species diversity and identifying biological specimens [7], and can supplement traditional taxonomic analyses by reducing the errors in species identification from morphological analysis [8].

Previously, ITS2 and *psbA-trnH* were considered the candidate barcodes for the interspecific identification among Paeoniaceae family [9,10]. Moreover, DNA barcoding has been studied for intraspecific [11,12]. We questioned whether they were suitable for the intraspecific identification of *P. lactiflora*. This study aimed to develop a taxonomic identification system to evaluate genetic diversity, conserving germplasm, and breeding traits.

2. Materials and methods

2.1. Plant materials

By standards of cut peony [13], twenty-one cut peony cultivars and the wild species were selected from the germplasm nursery of herbaceous peony, HeZe, Shandong, China (Table S1 and Fig. S1). All cultivars in this study have been authorized by China Flower Accession for naming specification in 2004 [14]. Under the stereoscope observation (XTL-850P) with magnification of 30 times, all cultivars with leaf edge spine were identified as intra-specific varieties of *P. lactiflora* [15] (Fig. 1).

2.2. DNA extraction, amplification, and sequencing

3–5 individuals were sampled and sequenced for each cultivars and their wild species. DNA extractions were conducted using the Plant Genomic DNA Kit (Tiangen Biotech Co., China). The universal primers

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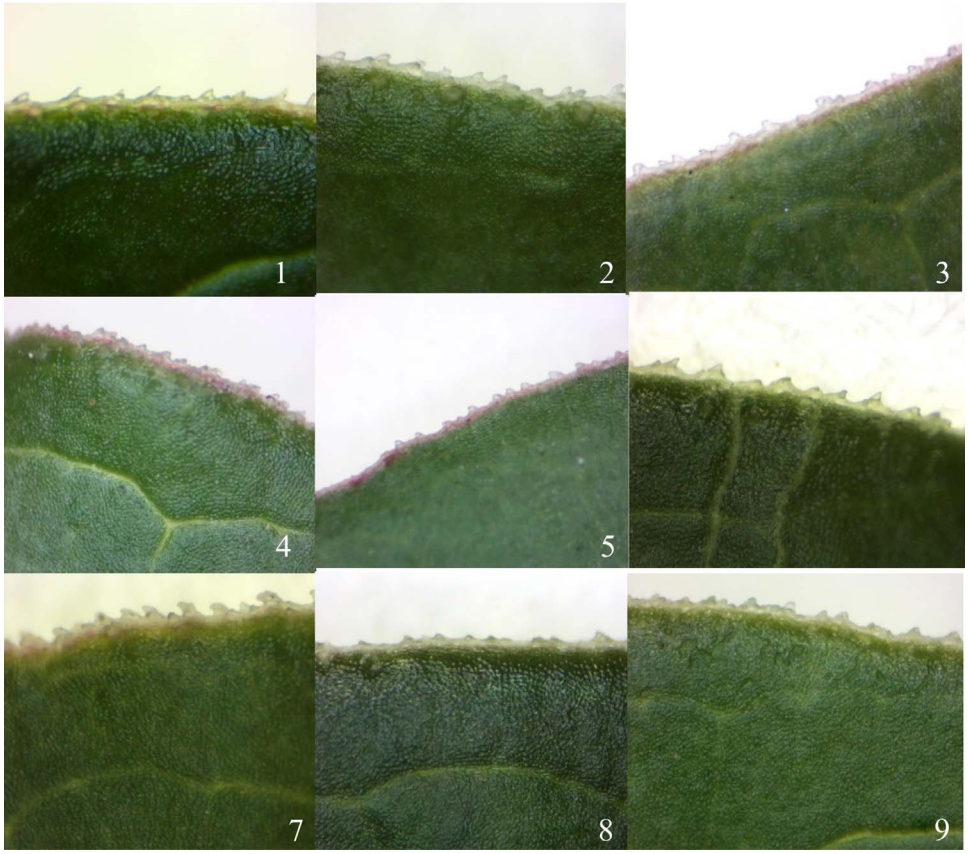

Fig. 1. Leaf morphology of partial cut peony cultivars under the stereoscope observation with magnification of 30 times. The cultivars used in this study (1). *P. lactiflora* (2). 'Qingtanlan' (3). 'Xueyuanhonghua' (4). 'Fushi'

(5).'Yangfeichuyu'(6).'Qihualushuang'(7).'Gaoganhong'(8).'Dongjingnvlang'(9). 'Fenchijinyu'.

Table 1
Sequencing primers and reaction conditions for ITS2 and *psbA-trnH*.

DNA marker	Primers Sequence(5′-3′)		PCR reaction conditions
ITS2	S2F	ATGCGATACTTGGTGTGAATTATAGAAT	94 °C 5 min, 94 °C 45s, 55 °C 45s,
	S3R	GACGCTTCTCCAGACTACAAT	72 °C 1 min, 35 cycles,72 °C 10 min
psbA-trnH	fwd PA	GTTATGCATGAACGTAATGCTC	95 °C 4 min, 94 °C 30s, 56 °C 1 min,
	Rev TH	CGCGCATGGTGGATTCACAATCC	72 °C 1 min, 35 cycles, 72 °C 10 min

for ITS2 and *psbA-trnH*, and general PCR reaction conditions were in Table 1. PCR amplification was performed in 25 µl reaction mixtures containing 30 ng of genomic DNA template, 2.5 µl 10 × PCR buffer with $MgCl_2$, 5.0 µM of each dNTP, 2.5 µM of each primer (synthesized by Sangon Co., China), and 1.0 U Taq DNA Polymerase. The PCR products were purified by TIANquick Midi Purification Kit (Tiangen Biotech Co., China) for ligation and transformed using pJET1.2 vector. Five to ten clones were screened in each cultivar; two of each were sequenced for both strands by an automated sequencer ABI Prism 3730 × l at Sangon Biotech Co., Ltd., Shanghai, China.

2.3. Genetic analysis and species identification

The raw sequencing results were corrected and assembled by CodonCode Aligner 3.0 (CodonCode Co., USA) to ensure sequencing accuracy. For ITS2, we used Hidden Markov Models (HMMS) [16] to delete possibly contaminated sequences from fungi. Sequence similarities were searched by BLASTn in Nucleotide Datebase, (GenBank), available at the National Center for Biotechnology Information. BLAST discriminates more accurately against sequences with high similarity [17–20]. E-value: Threshold for the best close match is 3.0%. Average intraspecific K2P genetic distances were calculated for ITS2 and *psbA-trnH* using MEGA6 software [21]. The secondary structures of ITS2 were predicted by the prediction tool in the ITS2 Database and the E-value cutoff was less than 1e-16 (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) [22–26].

2.4. Phylogenetic analysis

Phylogenetic relationship among the cultivars were analyzed from the alignment of sequences by ClustalW in MEGA6 [21], for constructing the phylogenetic trees, using MEGA6 software and UPGMA method on the basis of the K2P model with 50% deletion of gap/missing data. The bootstrap replication was set to 1000 to assess the reliability of phylogenetic trees. *P. suffruticosa* was used as an outgroup, whose accession numbers of ITS2 and *psbA-trnH* were U27692 and GQ435209, respectively.

3. Results

3.1. Evaluation of DNA barcodes

After amplification and sequencing, we obtained 22 ITS2 sequences and 18 *psbA-trnH* sequences. Their accession numbers were listed in Supplemental Table 1. The sequences lengths, GC content and other sequencing information are listed in Table 2. By Blastn, both loci

Table 2
Sequence information of two candidate barcodes for cut peony cultivars of *P. lactiflora*.

	ITS2	*psbA-trnH*
Number of samples	22	18
Length range (bp)	536	445
GC content (%)	52.16	33.49
Efficiency of PCR amplification (%)	100	100
Success rate of sequencing (%)	100	81.8
Average intra-species genetic distance	0.029	0.032
No.parsimony information/variable sites	25/100	17/138
Identification efficiency by Blast (%)	95.4	–
Identification efficiency by phylogenetic analysis (%)	63.6 (14/22)	61.1(11/18)

Note: '–' represents the failure of identification. For *psbA-trnH*, the samples have high similarity with uncertain species of *Paeonia* by Blastn.

correctly identified the samples at the genus level (see Table S2). However, ITS2 showed that most samples have 90% to 100% similarity with *P. lactiflora* with the exception of cultivar 'Huguangshanse' (HGSE) which has 99% similarity with *P. sinjiangensis*. For *psbA-trnH*, all samples displayed 94% to 99% similarity with *Paeonia* sp.

3.2. Secondary structure of ITS2-RNA

Free energy values of the ITS2 secondary structures ranged from −259.9 kJ/mol in cultivar 'Huguangshanse' (HGSE) to −321.9 kJ/mol in cultivar 'Chifen' (CF). The structures displayed some universal features: four helices which was the typical secondary structures for eukaryotes [27], a pyrimidine-pyrimidine bulge on helix II, and a UAAU sequence conserved on the 5′side of helix III (Fig. 2). The shortest helices (IV) was comparatively conservative, whereas others showed higher variability. The proportion of G-U pairs in the helices was generally low (see Table 3).

Nine types were distinguished by the morphometric characters of the helices I and III (Fig. 2, Table 3). Type I is identifies by having 18 base pairs in helix I. Type II is identified by having 20 base pairs in helix I and 31 base pairs in helix III. Type III is identified by having 22 base pairs in helix I and 31 base pairs in helix III. Type IV is identified by having 20 base pairs in helix I and 32 base pairs in helix III. Type V is identified by having 15 base pairs in helix I. Type VI is identified by having 22 base pairs in helix I and 28 base pairs in helix III. Type VII is identified by having 16 base pairs in helix I. Type VIII is identified by having 19 base pairs in helix I and 27 base pairs in helix III. Type IV is identified by having 19 base pairs in helix I and 31 base pairs in helix III. The secondary structure analysis was supported by the UPGMA analysis of ITS2.

3.3. Phylogenetic analysis

P. suffruticosa and *P. lactiflora* were in the *Paeonia* section and *Moutan* section, respectively. The UPGMA cluster analysis of ITS2 and *psbA-trnH* were shown in Figs. 3 and 4 For ITS2, the tree was separated into five clades. The first major subgroup consisted of the most cultivars and genetic distances among these cultivars were 0–0.0564, which was consistent with the morphological analysis. The out-group species was in the second subgroup alone. Cultivars 'Huguangshanse' (HGSS), 'Dongjingnvlang' (DDJNL), and 'Xueyuanhonghua' (XYHH) formed the third, fourth and fifth subgroup, respectively, which showed further genetic distances with others. Similarly for *psbA-trnH*, the phylogenetic tree was separated into three clades. Most cultivars grouped in the first major subgroup and the genetic distances among these cultivars were 0–0.0168. *P. suffruticosa* and cultivar 'Qingtianlan'(QTL) formed the second and third subgroup, respectively. As we observed, the clusters were not correlated with the flower colors and types.

4. Discussion

An ideal barcode marker should be universal for ease of amplification, sequencing, good sequence quality, and high discriminatory power [28]. In this study, ITS2 performed better in the amplification, sequencing and identification than *psbA-trnH* did, and satisfied the criteria of barcode.

For *psbA-trnH*, the presence of a poly-A/T in this region led to sequencing failure in some cultivars, which was the significant problem as a candidate DNA barcode for *P. lactiflora* cultivars. Moreover, the identification by Blastn at the species level was uncertain, making it unsuitable for *P. lactiflora* cultivars.

ITS2 has already been suggested as a potential DNA barcode for plants [10,29], but was previously reported as difficult to amplify and directly sequence due to the incomplete concerted evolution of the nuclear multiple-copy region caused by hybridization or other factors in some taxa [30]. Our results showed that ITS2 was easy to amplify and sequence. Shi-lin Chen tested the discrimination ability of ITS2 in more than 6600 plant samples belonging to 4800 species from 753 distinct genera and found that the rate of successful identification was 92.7% at the species level [10]. For Paeoniaceae, including 35 samples from 23 species, the successful identification rate at the genus level was 100%, but only 40% at the species level. In this study, intra-specific authentication efficiencies of ITS2 were 95.4% by Blastn and 63.6% by phylogenic analysis, which suggested that ITS2 was suitable for intra-specific classification of *P. lactiflora*.

Based on the leaf edge spine, all cultivars belonged to the intraspecific variations of *P. lactiflora* [15]. On the phylogeny tree of both loci, most cultivars grouped together with *P. lactiflora* and separated from *P. suffruticosa*, which is consistent with the taxon of *Paeonia*. However, several cultivars displayed closer genetic distances with *P. suffruticosa* than other cultivars, implied complex origin of these cultivars. Herbaceous peony varieties are primarily derived from *P. lactiflora*, native to northeast Asia [31–33], with the exception of Itoh hybrids, which was the intersectional hybrid peonies, using pollen of *P. suffruticosa* 'Alice Harding' on the *P. lactiflora* cultivar 'Kakoden' [34]. In China, the first intersectional hybrid peony 'Hexie' has been found [35]. Moreover, *Paeonia* is a phylogenetically and taxonomically complex group [36,37], especially for section *Paeonia*, which may have undergone complex reticulate evolution that further obscured phylogenetic relationships [38,39]. For the complex lineage of herbaceous peony cultivars, we estimate the *P. suffruticosa* may be involved in the lineage of some cut peony cultivars. Moreover, there is discordance between the phylogenies of ITS2 and *psbA-trnH*. Cultivars closer to *P. suffruticosa* on the phylogeny of ITS2 grouped with other cultivars on the phylogeny of *psbA-trnH*, which can be interpreted by the hybrid speciation which was the inheritance of cpDNA from one parent and fixation of ITS2 sequences from another parent [40,41]. Because maternal transmission of cpDNA has been found in the most of flowering plants, the parent whose cpDNA is transmitted to hybrid is very likely the maternal parent [42]. The discordance between the phylogeny of ITS2 and *psbA-trnH* may reflect the different maternal parent of these cultivars.

Herbaceous peony is not only a beautiful garden plant, but also an ornamental crop with an increasing economic importance in global floriculture. It has been popular as cut flowers. Cut peony flowers are highly valued in international market. Among the thousands of available cultivars, only a small number are suitable for fresh-cut flowers. Despite on-going breeding efforts in China, France, United States, and other countries, the most popular cultivars have been on the market for years [43]. Successful commercial production of new varieties is essential for breeders and researchers to satisfy the market. *P. lactiflora* cultivars inherited from ancestors in China where repeated hybridizations over thousands of years and the fluctuating habitats of wild plants have occurred abundant genetic diversity in this species [15,44], which will be good breeding materials. ITS2 identified the cut peony cultivars

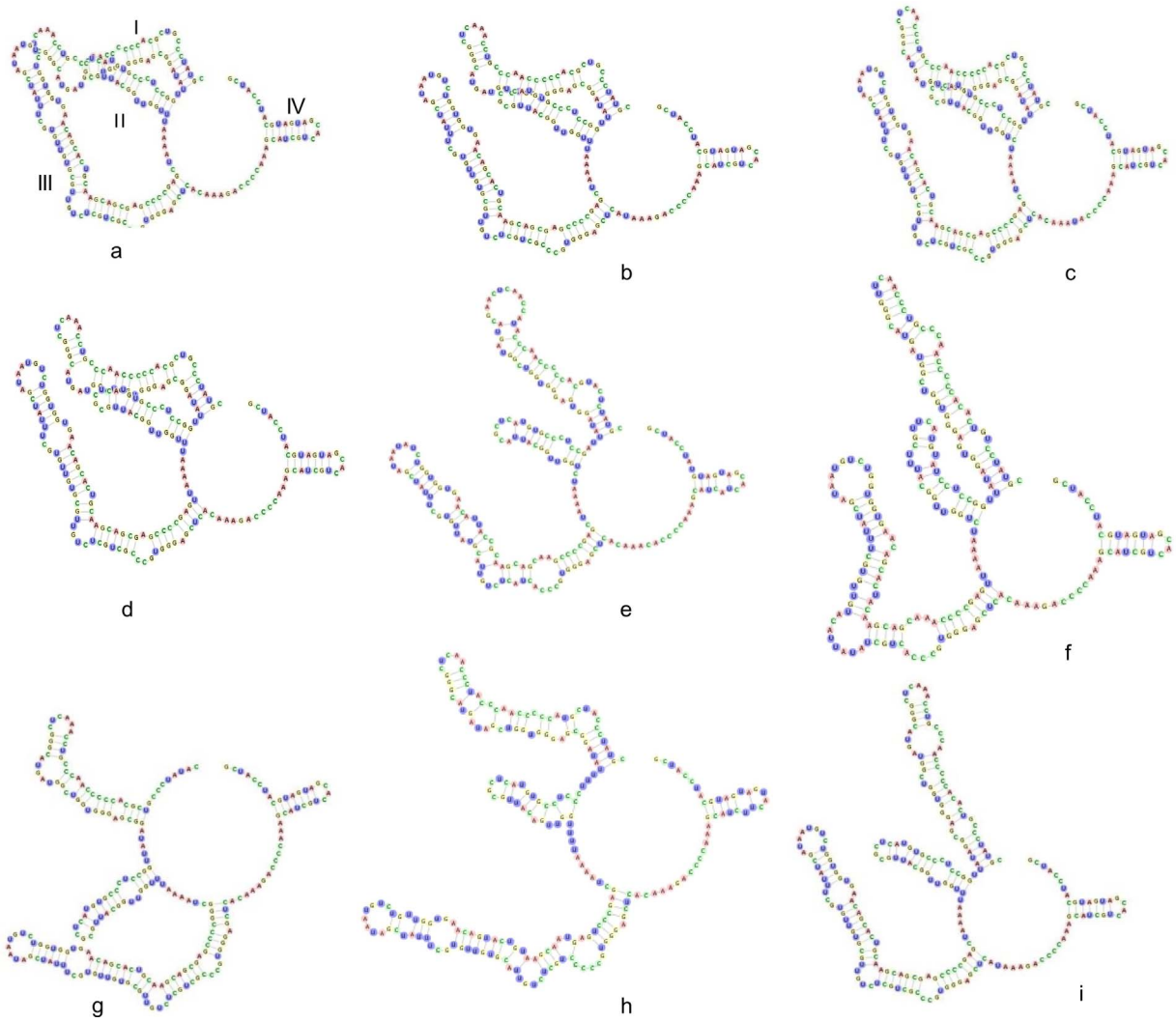

Fig. 2. ITS2 secondary structures of cut peony cultivars in ITS2 database. (a) Type I. (b) Type II. (c) Type III. (d) Type IV. (e) Type V. (f) Type VI. (g) Type VII. (h) Type VIII. (i) Type IX. *Note:* I –IV represent the helices of ITS2 secondary structures.

Table 3
The types of the secondary structures of ITS2 for cut peony cultivars of *P. lactiflora*.

Type	Cultivar(s)	G-U pairs	Helix length (bp)			
			I	II	III	IV
I	'Qingtianlan', 'Bingshan', 'Hongfushi'	9	18	9	31	7
II	'Fushi', 'Hongfeng', 'Huangjinlun', 'Fenchijinyu', 'Xuefeng' 'Gaoganhong', 'Tianshanhongxing', *P. lactiflora*	11	20	11	31	7
III	'Yangfenchuyu', 'Chifen'	10	22	11	31	7
IV	'Hongxiuqiu', 'Qihualushuang'	12	20	11	32	7
V	'Xueyuanhonghua'	8	15	9	25	5
VI	'Huguangshanse'	8	22	10	28	7
VII	'Guifeichacui', 'Dafugui', 'Qingwen'	12	16	11	31	7
VIII	'Dongjingnvlang'	8	19	8	27	6
IX	'Taohuafeixue'	12	19	11	31	7

and estimated the relationship among these cultivars, which will guide the future hybrid breeding. In conclusion, ITS2 enriches the bank of molecular markers available for cut peony cultivars and should be helpful for evaluating genetic diversity, conserving germplasm, and breeding desired traits.

5. Conclusion

In conclusion, we evaluated ITS2 for distinguishing *P. lactiflora* cultivars, which will be useful for intraspecific identification of *P. lactiflora* cultivars. Furthermore, phylogenetic analysis of ITS2 revealed that *P. lactiflora* cultivars may have evolved into different clades, indicated a non-monophyletic relationship between *P. lactiflora* and *P. suffruticosa*. In future, we will enlarge the samples and combine other taxon methods to investigate intraspecific relationship of herbaceous peony cultivars.

Conflict of interest

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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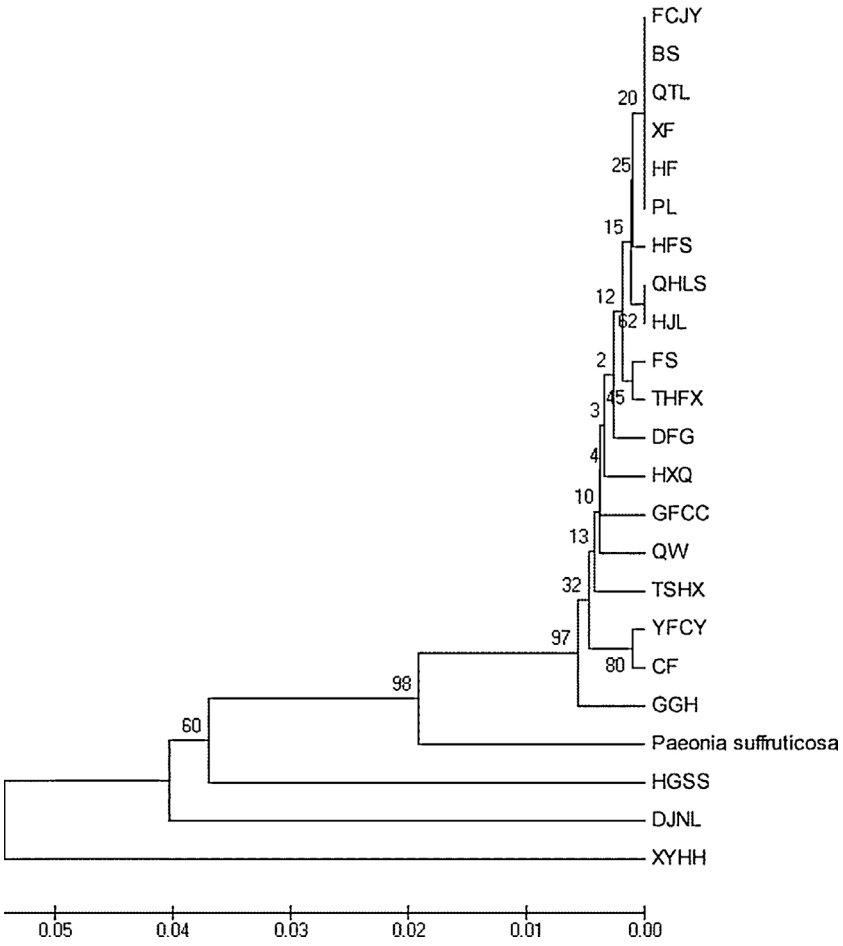

Fig. 3. UPGMA tree of pairwise K2P substitution rates of ITS2 for cut peony cultivars with 1000 replicates. Numbers on branch represent UPGMA support values (%).

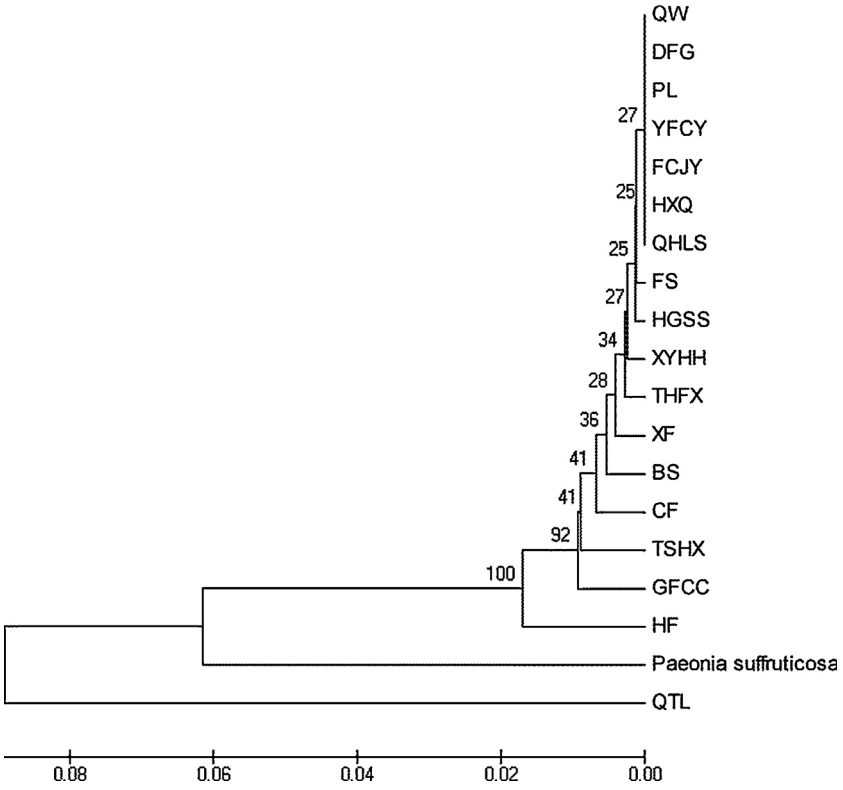

Fig. 4. UPGMA tree of pairwise K2P substitution rates of *psbA-trnH* for cut peony cultivars with 1000 replicates. Numbers on branch represent UPGMA support values (%).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2017.07.003.

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