Biotechnology Reports 15 (2017) 101–106

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

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

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ARTICLE INFO

Keywords:
Paeonia lactiflora
Intra-specific
DNA barcode

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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http://dx.doi.org/10.1016/j.btre.2017.07.003
Received 27 May 2017; Received in revised form 22 June 2017; Accepted 6 July 2017
Available online 08 July 2017

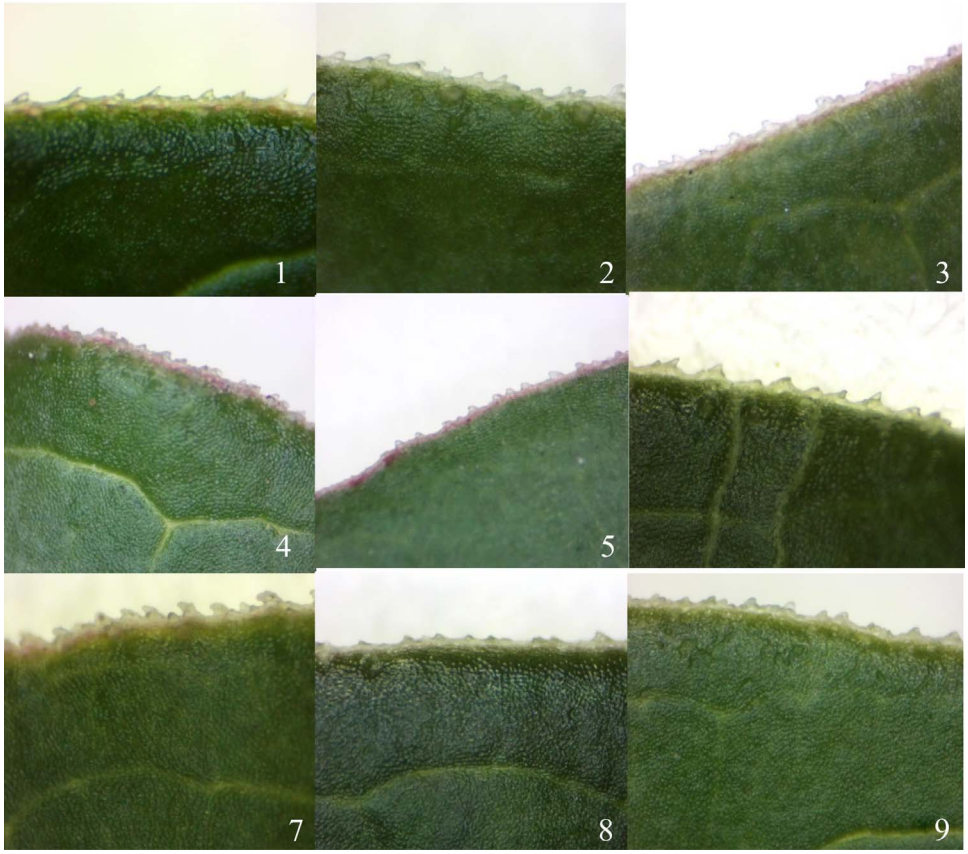

Fig. 1. Leaf morphology of partial cut peony cultivars under the stereoscope observation with magnification of 30 times. The cultivars used in this study (1). *P. lactiflora* (2). 'Qingtanlan' (3). 'Xueyuanhonghua' (4). 'Fushi'

(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