Identification of three kinds of Plumeria flowers by DNA barcoding and HPLC specific chromatogram

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ABSTRACT

DNA barcoding and HPLC specific chromatogram were used to identify three kinds of Plumeria flowers respectively. DNAs extracted from the three Plumeria species were amplified by PCR with universal primers, and the pbsA-trnH region was selected. All the amplified products were sequenced and the results were analyzed by MEGA 5.0. Chemometric methods including principal components analysis and hierarchical clustering analysis were conducted on the SAS 9.0 software to demonstrate the variability among samples. In conclusion, the pbsA-trnH of all samples were successfully amplified from total DNA and sequenced. These three varieties of Plumeria can be differentiated by the pbsA-trnH region and clustered into three groups respectively through building neighbor joining tree, which confirmed to their germplasm origins. However, it was hard to distinguish them by HPLC specific chromatograms combined with chemometrics analysis. These indicated that DNA barcoding was a promising and reliable tool for the identification of three kinds of Plumeria flowers compared to HPLC specific chromatogram generally used. It could be treated as a powerful complementary method for traditional authentication, especially for those varieties which are difficult to be identified by conventional chromatography.

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1. Introduction

Plumeria is a kind of deciduous tree belonging to Apocynaceae. It has a significant medicinal value and can be used for the treatment of various ailments, such as sore throat, heatstroke, belly-ache, cough, and dysentery [1]. The main chemical components of Plumeria are iridoids, triterpenes, flavonoids, essential oil and so on. Mansour et al. [2] found that the iridoids in Plumeria have a toxic effect on a series human tumor cells. The extracts of the flowers can significantly inhibit streptococcus faecalis, bacillus, and corynebacterium pyogenes [3]. Gupta et al. [4] indicated that the flowers extracts of Plumeria had the anti-typeric-analgesic effect. As a medicine with dual-purpose of drug and food, the flowers of Plumeria are also popular among people in making herbal tea to relieve the summer heat.

There are many varieties of Plumeria, such as Plumeria rubra L., Plumeria rubra var. alba, and Plumeria rubra ‘Acutifolia’. Some researches have indicated that the chemical components vary from different Plumeria flowers, some of which may have good bioactivities [5–8]. However, it is difficult to identify Plumeria flowers because they are very alike after dried and processed. Thus, authenticity assurance is crucial for their quality control. We intended to use DNA barcoding technique and HPLC specific chromatograms to identify three kinds of Plumeria.

DNA barcoding is a technique for identifying biological specimens using short DNA sequences from either nuclear or organelle genomes [9]. This technique has three advantages as follows. Firstly, the results have good repeatability compared to other molecular identification methods. Secondly, DNA barcoding technique is highly universal. The traditional taxonomic method requires that the leaves, flowers, fruits and other organs of plants must exist. However, DNA barcoding technique is not limited by growth stage, organ, tissue difference or the external environment. And a small number of samples are enough for the identification. Lastly, it can establish a unified database and identification platform to realize digital species identification [10]. DNA barcoding technique has been applied to identification of animals, gymnosperms, angiosperms, fungi and so on [11].

The chloroplast DNA (cpDNA) has been commonly used for DNA barcoding studies in plants. The cpDNA contains variable DNA regions, among which the most commonly used cpDNA intergenic spacer is psbA-trnH, which has shown high variability and can be used to elucidate genetic relationships at the intraspecific level [12,13]. In this pioneering study, we selected psbA-trnH to identify three varietas of Plumeria, at the same time, the HPLC specific
chromatogram that is commonly used for identification was also used for distinguishing these three varieties of *Plumeria* to make comparison. We hope that our established method will be helpful for the future quality control of *Plumeria* flowers.

## 2. Experimental

### 2.1. Materials and reagents

Twelve samples of *Plumeria* flowers were collected from three districts in Guangzhou, Guangdong, China (Table 1). They were authenticated by Associate Professor Lin Jiang, Sun Yat-Sen University, China. Methanol was of analytical grade and manufactured by Tianjin Zhiyuan Chemical Reagent Factory (Tianjin, China). TAE buffer, agarose, PVP-40 and *Taq* PCR Master Mix (2 ×, blue dye) were purchased from Sangon Biotech Shanghai, China). DNA secure Plant Kit was purchased from Sangon Guangzhou for total DNA extraction. PCR amplification was performed on the Shimadzu LC-15C column (250 mm × 4.6 mm, 5 μm; Dikma, Beijing, China) and a guard column (15 mm × 4.6 mm, 5 μm; Dikma, Beijing, China).

### 2.2. Apparatus

An electronic balance (KERN ABT 220-5DM, 0.1 mg, Germany), an ultrasonic machine (SB25-12DTD, Xinzhi Biotechnical Ltd., Ningbo, China) and an eppendorf centrifuge 5417R (Eppendorf AG, Hamburg, Germany) were used for sample preparation and DNA extraction. PCR amplification was performed on the K960 thermal cycler (Hangzhou Jingge Scientific Instrument Co., Ltd, Hangzhou, China). HPLC analysis was performed on the Shimadzu LC-15C high performance liquid chromatograph (Shimadzu, Japan) with a Dikma Diamonsil C18 column (250 mm × 4.6 mm, 5 μm; Dikma, Beijing, China) and a guard column (15 mm × 4.6 mm, 5 μm; Dikma, Beijing, China).

### 2.3. Sample pre-treatment for DNA extraction and total DNA extraction

The fresh flowers were washed up and wiped with 75% alcohol aqueous. About 100 mg of each sample was grinded with 1% (m/m) PVP-40. Then, DNA secure Plant Kit was used for total DNA extraction. The process followed the instruction of the Kit.

### 2.4. PCR amplification and DNA sequencing

DNA barcodes were amplified by polymerase chain reaction using universal primers (fwd: 5′-GGTATGCATGACCGTATCC-3′ and rev: 5′-CCGCATGGATCCCAAATCC-3′). Each 25 μL reaction mixture contained 12.5 μL *Taq* PCR Master Mix, 1 μL Genomic DNA, 1 μL of each 10 μM primer, 1 μL MgC2 solution and 8.5 μL ddH2O.

The PCR conditions for amplification were 1 cycle 94 °C for 5 min; 40 cycles of 94 °C 30 s, 56 °C 30 s, 72 °C 45 s; and 1 cycle 72 °C for 10 min, and hold 4 °C. To detect amplified products successfully, PCR products were examined on 2% agarose gels stained with Goldview and visualized under ultraviolet light.

### 2.5. Sequence alignment and analysis

All the amplified products were sent to Sangon Guangzhou for sequencing. The sequences were analyzed by MEGA 5.0.

### 2.6. Preparation of sample solution for HPLC analysis

Test solutions were prepared by extracting 0.5 g dried and pulverized herbs with 10 mL 70% methanol aqueous under ultrasonic condition at room temperature for 30 min. After cooling, the extracted solution was added with 70% methanol aqueous to the original weight. The extracts were filtered through a 0.45 μm filter before used for HPLC analysis.

### 2.7. HPLC conditions

Chromatographic separation was carried out on a Diamonsil C18 column along with a guard column. The separation was conducted at 35 °C with a flow rate of 0.7 mL/min. 0.5% acetic acid aqueous solution (A) and acetonitrile (B) were used as the mobile phase in gradient elution mode. The elution gradient was set as follows: 0–10 min, 10% (B); 10–30 min, 10% → 15% (B); 30–70 min, 15% → 20% (B); 70–85 min, 20% → 65% (B); 85–90 min, 65% → 90% (B); 90–95 min, 90% (B). The detection wavelength was 240 nm. The injection volume was 20 μL.

### 2.8. Chemometric analysis

Principal components analysis (PCA) and hierarchical clustering analysis (HCA) were conducted on the SAS 9.0 software to demonstrate the variability among the 12 samples.

## 3. Results and discussion

### 3.1. DNA barcode result analysis

Authenticity assurance is crucial for quality control of natural products. It is essential to develop different approaches to authenticate the natural products as each approach has advantages that complementary to one another [14]. A desirable DNA barcode

| Samples | Latin name | District          |
|---------|------------|------------------|
| R1      | Plumeria rubra L. | Sun Yat-Sen University campus | Panyu Guangzhou |
| R2      | Plumeria rubra L. | Sun Yat-Sen University campus | Panyu Guangzhou |
| R3      | Plumeria rubra L. | Sun Yat-Sen University campus | Panyu Guangzhou |
| R4      | Plumeria rubra L. | Sun Yat-Sen University campus | Panyu Guangzhou |
| P1      | Plumeria rubra var. alba | Tianhe Park | Tianhe Guangzhou |
| P2      | Plumeria rubra var. alba | Tianhe Park | Tianhe Guangzhou |
| P3      | Plumeria rubra var. alba | Tianhe Park | Tianhe Guangzhou |
| P4      | Plumeria rubra var. alba | Tianhe Park | Tianhe Guangzhou |
| W1      | Plumeria rubra ‘Acutifolia’ | Sun Yat-Sen University campus | Panyu Guangzhou |
| W2      | Plumeria rubra ‘Acutifolia’ | Sun Yat-Sen University campus | Panyu Guangzhou |
| W3      | Plumeria rubra ‘Acutifolia’ | Sun Yat-Sen University campus | Panyu Guangzhou |
| W4      | Plumeria rubra ‘Acutifolia’ | Sun Yat-Sen University campus | Panyu Guangzhou |
should process high interspecific divergences and low intraspecific variations. The Consortium for the Barcode of Life (CBOL) suggested comparing the interspecies distance ($d_{\text{inter}}$) and intraspecies distance ($d_{\text{intra}}$) to estimate the identification effectiveness of the selected barcode. The interspecific divergences of an ideal ‘barcoding gap’ should be significantly larger than intraspecific divergences. If $d_{\text{inter}}/d_{\text{intra}}$ is smaller than 1, it may not be a suitable DNA barcode [10].

The results of DNA barcoding showed a good differentiation. The psbA-trnH of all samples were successfully amplified from total DNA and sequenced. Properties of the psbA-trnH region are summarized in Table 2. The genetic distance was calculated by MEGA 5.0, based on Kimura-2-parameter model. The intraspecies distances of Plumeria rubra L. and Plumeria rubra ‘Acutifolia’ were 0.001 and 0.004, respectively. There was no intraspecies distance for Plumeria rubra var. alba. The interspecies distance was 0.007 between Plumeria rubra L. and Plumeria rubra var. alba, 0.034 between Plumeria rubra L. and Plumeria rubra ‘Acutifolia’, 0.033 between Plumeria rubra var. alba and Plumeria rubra ‘Acutifolia’. Results of each $d_{\text{inter}}/d_{\text{intra}}$ were larger than 1, which indicated that the psbA-trnH region was suitable for the identification of three varietas of Plumeria. The neighbor joining tree was built by MEGA 5.0 by repeated 1000 times bootstrap (Fig. 1). The neighbor joining tree showed that the samples of Plumeria rubra L., Plumeria rubra var. alba and Plumeria rubra ‘Acutifolia’ can be clustered into three groups, respectively. Therefore, the psbA-trnH region was an appropriate DNA barcode for identifying these three varietas of Plumeria.

3.2. HPLC analysis

Fig. 2 shows the HPLC specific chromatograms of samples R1 (Plumeria rubra L.), P1 (Plumeria rubra var. alba) and W1 (Plumeria rubra ‘Acutifolia’), their HPLC chromatograms were so similar that it was difficult to separate the three varietas visually.

3.3. Principal component analysis (PCA)

PCA, a multivariate analysis technique, could visualize similarities or differences within multivariate data [15]. It was employed to analyze the differences among these 12 samples. The peak areas of 6 characteristic peaks were set as variables, while 12 samples were set as observations. PC1 explained 41.2% of the total variance in the data set while PC2 explained 32.4%. The cumulative
proportion of PCA as well as the loading diagram is shown in
Fig. 3. According to the loading diagram, PC1 showed a strong
correlation with peak 5 and peak 6. PC2 showed a strong corre-
lation with peak 2 and peak 4. PC3 showed a strong correlation
with peak 1 and peak 5. The distinguished results of HPLC specific
chromatograms combined with PCA were not as accurate as that of
DNA barcoding.

3.4. Hierarchical cluster analysis (HCA)

HCA, one of the most commonly used unsupervised pattern
recognition methods, is a useful multivariate statistic technique
to assign a data set into groups by creating a cluster tree or
dendrogram according to similarity [15]. In order to assess the
resemblance and differences of these samples, HCA of Plumeria
samples was performed based on the peak area of the 6 char-
acteristic chromatographic peaks by SAS 9.0 software. The Ward’s
method was applied as the amalgamation rule and the squared
Euclidean distance was selected to measure the resemblance and
classify the 12 samples. The result is shown in Fig. 4. Samples R1–
R4 (Plumeria rubra L.) were categorized into one cluster, W1–W4
(Plumeria rubra ‘Acutifolia’) were categorized into another clus-
ter. However, samples P1–P4 (Plumeria rubra var. alba) cannot
cluster together. Samples R1–R4 and W1–W4 were collected from
the same district, but samples P1–P4 were from three different
districts. Therefore, we speculated that the environment affected
the chemical component which affected the result of PCA
and HCA.

4. Conclusion

This study has shown that DNA barcoding combined with
chemometrics analysis can distinguish Plumeria rubra L., Plumeria
rubra ‘Acutifolia’ and Plumeria rubra var. alba. while the HPLC
method cannot identify these three varieties because the specific
chromatograms were similar. DNA barcoding technique is hopeful
for automation as it is more stable, accurate and not affected by
growth stage, tissue difference or external environment. This
technique is an effective supplement for traditional authentication
methods, especially when different species are mixed together.
This work also provides an experimental reference for identifica-
tion of natural medicines by DNA barcoding technique.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

[1] Chinese Herbal Medicines Guangxi Standard, 1st volume, The Guangxi Zhuang
Autonomous Region Health Department, Guangxi Science and Technology
Press, Nanning, 1990: 59.
[2] A.K. Mansour, M.M. Eid, N.S.A.M. Khalil, Synthesis and reactions of some new
heterocyclic carbohydrazides and related compounds as potential anticancer
agents, Molecules 8 (2003) 744–755.
[3] P.A. Egwaikhide, S.O. Okeniyi, C.E. Ginda, Screening for anti-microbial activity
and phytochemical constituents of some Nigerian medicinal plants, J. Med.
Plants Res. 3 (2009) 1088–1091.
[4] M. Gupta, U.K. Mazumder, P. Gomathi, Evaluation of antipyretic and anti-
nociceptive activities of Plumeria acuminata leaves, J. Med. Sci. 7 (2007)
452–456.
[5] Z. Zahee, A.G. Konale, K.A. Patel, et al., Comparative phytochemical screening
of flowers of Plumeria alba and Pulmeric rubra, Asian J. Pharm. Clin. Res. 3
(2010) 88–89.
[6] N. Tohar, M.A. Mohd, I. Jantan, et al., A comparative study of the essential oils
of the genus Plumeria Linn, from Malaysia, Flavour Fragr. J. 6 (2006) 304–309.
[7] N. Tohar, K. Awang, M.A. Mohd, et al., Chemical composition of the essential oils of four Plumeria specials grown on Peninsular Malaysia, J. Essent. Oil Res. 6 (2006) 613–617.

[8] Y.Q. Liu, H.W. Wang, S. Wei, et al., Chemical composition and antimicrobial activity of the essential oils extracted by microwave-assisted hydrodistillation from the flowers of two Plumeria species, Anal. Lett. 45 (2012) 2389–2397.

[9] N. Techen, I. Parveen, Z.Q. Pan, et al., DNA barcoding of medicinal plant material for identification, Curr. Opin. Biotechnol. 25 (2014) 103–110.

[10] S.L. Chen, DNA Barcoding Molecular Markers in Chinese Medicinal Materials, People’s Medical Publishing House, Beijing, 2012: 18–19.

[11] S.L. Chen, X. Hui, J.P. Han, et al., Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species, PLoS One 5 (2010) e8613.

[12] H. Azum, J.C. García-Franco, V. Rico-Gray, et al., Molecular phylogeny of the Magnoliaceae: the biogeography of tropical and temperate disjunctions, Am. J. Bot. 88 (2001) 2275–2285.

[13] M.B. Hamilton, J.M. Braverman, D.F. Soria-Hernanz, Patterns and relative rates of nucleotide and insertion/deletion evolution at six chloroplast intergenic regions in new world species of the Lecythidaceae, Mol. Biol. Evol. 20 (2003) 1710–1721.

[14] P.C. Shaw, J. Wang, P.P.H. But, Authentication of Chinese Medicinal Materials by DNA Technology, World Scientific, Singapore, 2002: 1–23.

[15] Z.S. Xie, S.Z. Lam, J.W. Wu, et al., Chemical fingerprint and simultaneous determination of flavonoids in Flos Sophorae Immaturus by HPLC-DAD and HPLC-DAD-ESI-MS/MS combined with chemometrics analysis, Anal. Methods 6 (2014) 4328–4335.