

Identification of medical plants of 24 *Ardisia* species from China using the *matK* genetic marker

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ABSTRACT

Background: *Ardisia* is a group of famous herbs in China, which has been used as medical plants for more than 900 years. However, the species from the genus are so analogous that it is difficult to discriminate them just by morphological characteristics. DNA barcoding is a new technique that uses a short and standard fragment of DNA sequences to identify species. **Objective:** Choose a suitable DNA marker to authenticate *Ardisia* species. **Materials and Methods:** Four markers (*psbA-trnH*, internal transcribed spacer 2 [ITS2], *rbcl*, *matK*) were tested on 54 samples of 24 species from genus *Ardisia*. The success rates of polymerase chain reaction amplification and sequencing, differential intra- and inter-specific divergences, DNA barcoding gap and identification efficiency were used to evaluate the discrimination ability. **Results:** The results indicate that *matK* has the highest interspecific divergence and significant differences between inter- and intra-specific divergences, whereas *psbA-trnH*, ITS2 and *rbcl* have much lower divergence values. *Matk* possessed the highest species identification efficiency at 98.1% by basic local alignment search tool 1 [BLAST1], method and 91.7% by the nearest distance method. **Conclusion:** The *matK* region is a promising DNA barcode for the genus *Ardisia*.

Key words: *Ardisia*, DNA barcoding, identification, *matK*

INTRODUCTION

Ardisia genus is a group of flowering plants belonging to *Myrsinaceae* family, native to tropical American, Austronesia, India Peninsula, East and South Asian, minority spread over Oceania. The genus includes about 300 species in the world and 68 species in China, which is widely and commonly cultivated in south area of Yangtze River.^[1] Most species of *Ardisia* are medicinal plants and a few of them are ornamental plants in China. Some of them are famous on medicinal value. For example, *Ardisia japonica* (Hornst.) Blume is commonly used for treating chronic bronchitis; *Ardisia crenata* Sims var. *crenata* is used as oxytocics and anti-pregnancy drugs. *Ardisia pusilla* A. de Candolle. is used to treat traumatic injuries.^[2] But the species from the genus are so analogous that it is very difficult to discriminate them just by morphological characteristics and it is often taken place that many species of the genus are confused and used by other different

species. So, it is very important to accurately identify these medical plants from *Ardisia*.

DNA barcoding, which was first proposed by Hebert *et al.*^[3] is a new technique that uses a short and standardized fragment of DNA sequences to identify species, and recently it has become a hotspot of biodiversity research.^[4] In subsequent research,^[5-7] Hebert *et al.* found that the CO1 gene is a standard DNA barcode for animals. But the studies on plant barcodes are much more complicated than that of animals, because of the hybridization and reticulate evolutionary histories.^[8,9] Recently, a number of single loci and combined loci have been suggested as candidate barcode sequences for plant identification,^[10-12] but there was no consensus on universal DNA barcode for all plant species. For every concrete group of species, especially those which contain many closely related species, applicable loci have to be studied and choose. Some scholars have done DNA barcoding researches in related species and genera, but no one has evaluated feasibility of the method in plants of *Ardisia*.

In this context, we choose four regions intensively recommended (*psbA-trnH*, *matK*, *rbcl*, internal transcribed

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spacer 2 [ITS2]) to test and evaluate the feasibility of these regions as candidate DNA barcodes to discriminate medicinal species in China from *Ardisia* and try to find a new a digital identification method for medicinal plants of *Ardisia*.

MATERIALS AND METHODS

Plant materials

The experimental samples were collected from (1) South China Botanical Garden, Guangdong Research Institute of Traditional Chinese Medicine, Guangdong province, and authenticated by Prof. Yuewen Cai of the Institute; (2) Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Yunnan province, and authenticated by senior Engineer Chunfen Xiao of the Garden; (3) Wuhan Botanical Garden, Chinese Academy of Sciences, Hubei province, and authenticated by Engineer Shouzhong Zhang of the Garden. All voucher images and specimens were deposited in the herbarium of Hubei University of Chinese Medicine. The information of 54 samples belonged to 27 species are given in Table 1.

DNA extraction, amplification, and sequencing

First, leaf tissues were dried in silica gel. A total of 10 mg of each of the dried tissues was rubbed for 1 min at a frequency of 30 times/s in a FastPrep bead mill (Retsch MM400, Germany). Total DNA was extracted using the Plant Genomic DNA Kit (Tiangen Biotech Co., China). The polymerase chain reaction (PCR) reaction mixture consisted of 2 μ L (~60 ng) DNA, 4 μ L of 25 mM MgCl₂, 5 μ L of 10 × PCR buffer, 2U of Taq DNA polymerase, 4 μ L of 2.5 mM deoxy-ribonucleoside triphosphates [dNTPs] mix (Biocolor BioScience & Technology Co., China), 2.0 μ L 2.5 μ M of primers (Synthesized by Sangon Co., China), the final volume was 50 μ L. The sequences of the universal primers for the DNA barcode to be tested and general PCR reaction conditions were obtained from previous studies by Chen *et al.*^[13] PCR products were first examined with 1.5% agarose gel electrophoresis and purified using the Gel Band Purification Kit (Tiangen Biotech Co., China) and then sequenced in both directions with the primers used for PCR amplification on a 3730XL sequencer (Applied Biosystems, USA). The sequences were submitted to GenBank [Table 1].

Data analyses

The original forward and reverse sequences were assembled and edited using CodonCode Aligner 3.0 (CodonCode Co., USA) to estimate the quality of the generated sequence traces. Sequences alignment and checking were conducted by Clustal W. The ITS2 sequences were retrieved according

to Keller *et al.*^[14] and other sequences were retrieved using CodonCode Aligner. All the experimental materials were used to investigate the amplification efficiency of each sequence. The inter/intra-specific variation of the samples was calculated according to Luo *et al.*^[15] and Zhu *et al.*^[16] and Wilcoxon signed rank tests^[17] were used to check the result. DNA barcoding gap was produced using Taxon DNA.^[18] After the data from GenBank database were brought into, basic local alignment search tool 1 [BLAST1] and the nearest distance method were performed as described previously^[19] to assess the identification efficiency of each candidate sequence.

RESULTS

PCR amplification efficiency and the success rate of sequencing

The efficiency of PCR amplification and the success rate of sequencing of the four candidates were compared. The result showed that the efficiency of PCR amplification of *rbcL*, *psbA-trnH*, ITS2 and *matK* region were 100%, 100%, 100% and 88.9%. And they were all successfully sequenced by 100% [Table 2]. The sequence length, Guanine and Cytosine [GC] content of the four regions based on the results of the CodonCode Aligner and Clustal W alignment were presented [Table 2].

The analysis of intra-specific variations and inter-specific divergences

An ideal barcode should show the low intra-specific variations and high inter-specific divergences in order to distinguish different species. Here, six parameters were used to characterize inter-specific versus intra-specific variation [Table 3]. Through comparison of interspecific genetic distances among congeneric species for four candidate barcode, ITS2 region exhibited the highest interspecific divergence with all four metrics, followed by *psbA-trnH* and *matK*, while *rbcL* provided the lowest [Table 3]. We also found that *rbcL* showed the lowest level of intraspecific variation with all four metrics, followed by *psbA-trnH* and *matK*, while ITS2 provided the highest [Table 3].

Validation of the different sequences' inter/intra-specific variation

The results of Wilcoxon signed rank tests confirmed that *matK* provided much higher inter-specific divergence among congeneric species [Table 4] and the higher variation between conspecific individuals [Table 5].

Assessment of barcoding gap

Barcodes should exhibit a “barcoding gap” between interspecific and intraspecific distances.^[17,20] Although the histogram did not show a clear gap between intraspecific

Table 1: The collection sites and GenBank accession of 54 samples of the *Ardisia* genus

Species name ^a	Collection sites ^b	Voucher number	Gen Bank accession			
			ITS2	<i>psb A-trnH</i>	<i>rbcL</i>	<i>matK</i>
<i>A. chinensis</i> Benth.	1	KS0401MT01	JN252968	JN253070	JN253124	JN253022
<i>A. dasyrhizomatica</i> C. Y. Wu et C. Chen	1	KS0402MT01	JN252969	JN253071	JN253125	JN253023
<i>A. dasyrhizomatica</i> C. Y. Wu et C. Chen	1	KS0402MT02	JN252970	JN253072	JN253126	
<i>A. squamulosa</i> Presl.	1	KS0403MT01	JN252971	JN253073	JN253127	JN253024
<i>A. squamulosa</i> Presl.	1	KS0403MT02	JN252972	JN253074	JN253128	JN253025
<i>A. squamulosa</i> Presl.	1	KS0403MT03	JN252973	JN253075	JN253129	JN253026
<i>A. squamulosa</i> Presl.	2	KS0403MT04	JN252974	JN253076	JN253130	JN253027
<i>A. curvula</i> C. Y. Wu et C. Chen	1	KS0404MT01	JN252975	JN253077	JN253131	JN253028
<i>A. corymbifera</i> var. <i>tuberifera</i> C. Chen	1	KS0405MT01	JN252976	JN253078	JN253132	JN253029
<i>A. humilis</i> Vahl	1	KS0406MT01	JN252977	JN253079	JN253133	JN253030
<i>A. humilis</i> Vahl	1	KS0406MT02	JN252978	JN253080	JN253134	JN253031
<i>A. humilis</i> Vahl	3	KS0406MT03	JN252979	JN253081	JN253135	JN253032
<i>A. humilis</i> Vahl	3	KS0406MT04	JN252980	JN253082	JN253136	JN253033
<i>A. virens</i> Kurz var. <i>virens</i>	1	KS0407MT01	JN252981	JN253083	JN253137	JN253034
<i>A. virens</i> Kurz var. <i>virens</i>	1	KS0407MT02	JN252982	JN253084	JN253138	JN253035
<i>A. virens</i> Kurz var. <i>virens</i>	4	KS0407MT03	JN252983	JN253085	JN253139	
<i>A. crispa</i> (Thunb.) A. DC. var. <i>crispa</i>	1	KS0408MT01	JN252984	JN253086	JN253140	JN253036
<i>A. crenata</i> Sims var. <i>crenata</i>	5	KS0409MT01	JN252985	JN253087	JN253141	JN253037
<i>A. crenata</i> Sims var. <i>crenata</i>	5	KS0409MT02	JN252986	JN253088	JN253142	JN253038
<i>A. crenata</i> Sims var. <i>crenata</i>	1	KS0409MT03	JN252987	JN253089	JN253143	JN253039
<i>A. crenata</i> Sims var. <i>crenata</i>	1	KS0409MT04	JN252988	JN253090	JN253144	JN253040
<i>A. crenata</i> Sims var. <i>crenata</i>	3	KS0409MT05	JN252989	JN253091	JN253145	JN253041
<i>A. crenata</i> Sims var. <i>crenata</i>	3	KS0409MT06	JN252990	JN253092	JN253146	JN253042
<i>A. virens</i> Kurz var. <i>annamensis</i> Pitard	1	KS0410MT01	JN252991	JN253093	JN253147	JN253043
<i>A. polycephala</i> Wall. ex A. DC.	1	KS0411MT01	JN252992	JN253094	JN253148	JN253044
<i>A. japonica</i> (Thunb.) Blume	1	KS0412MT01	JN252993	JN253095	JN253149	JN253045
<i>A. japonica</i> (Thunb.) Blume	2	KS0412MT02	JN252994	JN253096	JN253150	JN253046
<i>A. japonica</i> (Thunb.) Blume	2	KS0412MT03	JN252995	JN253097	JN253151	JN253047
<i>A. elegans</i> Andr.	1	KS0413MT01	JN252996	JN253098	JN253152	JN253048
<i>A. elegans</i> Andr.	3	KS0413MT02	JN252997	JN253099	JN253153	JN253049
<i>A. crenata</i> Sims var. <i>bicolor</i> (Walker) C. Chen	1	KS0414MT01	JN252998	JN253100	JN253154	JN253050
<i>A. pusilla</i> A. DC.	3	KS0415MT02	JN252999	JN253101	JN253155	JN253051
<i>A. pusilla</i> A. DC.	4	KS0415MT03	JN253000	JN253102	JN253156	JN253052
<i>A. pusilla</i> A. DC.	4	KS0415MT04	JN253001	JN253103	JN253157	
<i>A. fordii</i> Hemsl.	1	KS0416MT01	JN253002	JN253104	JN253158	JN253053
<i>A. quinquegona</i> Bl. var. <i>quinquegona</i>	2	KS0417MT01	JN253003	JN253105	JN253159	JN253054
<i>A. quinquegona</i> Bl. var. <i>quinquegona</i>	2	KS0417MT02	JN253004	JN253106	JN253160	JN253055
<i>A. quinquegona</i> Bl. var. <i>quinquegona</i>	3	KS0417MT03	JN253005	JN253107	JN253161	JN253056
<i>A. quinquegona</i> Bl. var. <i>quinquegona</i>	3	KS0417MT04	JN253006	JN253108	JN253162	JN253057
<i>A. punctata</i> Lindl.	2	KS0418MT01	JN253007	JN253109	JN253163	JN253058
<i>A. punctata</i> Lindl.	2	KS0418MT02	JN253008	JN253110	JN253164	JN253059
<i>A. ordinata</i> Walker	2	KS0419MT01	JN253009	JN253111	JN253165	JN253060
<i>A. mamillata</i> Hance	3	KS0420MT01	JN253010	JN253112	JN253166	JN253061
<i>A. densilepidotula</i> Merr.	3	KS0421MT01	JN253011	JN253113	JN253167	JN253062
<i>A. densilepidotula</i> Merr.	3	KS0421MT02	JN253012	JN253114	JN253168	JN253063
<i>A. hanceana</i> Mez	3	KS0422MT01	JN253013	JN253115	JN253169	JN253064
<i>A. hanceana</i> Mez	3	KS0422MT02	JN253014	JN253116	JN253170	JN253065
<i>A. triflora</i> Hemsl.	4	KS0423MT01	JN253015	JN253117	JN253171	
<i>A. triflora</i> Hemsl.	4	KS0423MT02	JN253016	JN253118	JN253172	JN253066
<i>A. corymbifera</i> Mez var. <i>corymbifera</i>	4	KS0424MT01	JN253017	JN253119	JN253173	JN253067
<i>A. corymbifera</i> Mez var. <i>corymbifera</i>	4	KS0424MT02	JN253018	JN253120	JN253174	JN253068
<i>A. primulaefolia</i> Gardn. et Champ.	4	KS0425MT01	JN253019	JN253121	JN253175	JN253069
<i>A. maculosa</i> Mez var. <i>maculosa</i>	4	KS0426MT01	JN253020	JN253122	JN253176	
<i>A. ensifolia</i> Walker	4	KS0427MT01	JN253021	JN253123	JN253177	

^a *Ardisia*: A. ¹Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Yunnan, China; ² Guangdong Research Institute of Traditional Chinese Medicine, Guangdong, China; ³ South China Botanical Garden, Guangdong, China; ⁴ Wuhan Botanical Garden, Chinese Academy of Sciences, Hubei, China; ⁵ Kunming Institute of Botany, Yunnan, China; ITS2: Internal transcribed spacer 2

variation and interspecific divergence in the distributions of the four tested loci (*matK*, *rbcL*, ITS2, *psbA-trnH* intergenic spacer) [Figure 1], the results of Wilcoxon two-sample tests showed that the distribution of inter-specific divergences for the four barcodes were higher than that of intra-specific variations [Table 6]. All the four candidate sequences showed significant difference ($P < 0.05$).

Evaluation of identifying ability of barcodes

Two methods of species identification, including BLAST1 and the nearest distance method were used to test the

applicability of using different regions for unique species identification. In the BLAST1 method, the results showed that the *matK* region identified correctly 98.1% of the samples at the species level. In contrast to *matK*, the identification efficiency of *psbA-trnH*, ITS2 and *rbcL* were much lower at the species level. The results confirmed that *matK* had the highest success rate at the species level identification with both two methods [Table 7].

DISCUSSION

The screening of DNA barcode for the Ardisia genus

Optimal DNA barcode should meet following requirements: (1) Significant inter-species variance; (2) Sufficient small intra-species variance; (3) It should be amplified by single primer and have quality sequence by dual sequencing.^[21] In this research, we tested four DNA regions (*psbA-trnH*, ITS2, *rbcL* and *matK*) using 55 plant samples belonging to 27 closely related species from the *Ardisia* genus.

PsbA-trnH fragment has one of the biggest evolution rate among chloroplast compartment and flanked with approximate 75 bp conservative sequences at two ends, which can be used for designing universal primer.^[8,11,22]

Table 2: Success rate of sequencing, length range, GC content

Markers	<i>psbA-trnH</i>	ITS2	<i>rbcL</i>	<i>matK</i>
Number of samples /n	54	54	54	54
Success of sequencing /n	54	54	54	48
Success rate of sequencing/%	100	100	100	88.9
Length range/bp	439-494	219-225	717	928-966
GC content/%	0.292	0.610	0.430	0.332

ITS2: Internal transcribed spacer 2; GC: Guanine and Cytosine; Bp: Base pair

Table 3: Analysis of inter-specific divergence between congeneric species and intra-specific variation for the whole sample

Markers	<i>psbA-trnH</i>	ITS2	<i>matK</i>	<i>rbcL</i>
All inter-specific distance	0.0162±0.0098	0.0368±0.0159	0.0159±0.0074	0.0044±0.0028
Theta prime	0.0142±0.0065	0.0358±0.0133	0.0158±0.0040	0.0041±0.0017
Minimum inter-specific distance	0.0033±0.0056	0.0154±0.0157	0.0048±0.0035	0.0014±0.0018
All intra-specific distance	0.0053±0.0064	0.0119±0.0155	0.0056±0.0052	0.0009±0.0017
Theta	0.0069±0.0084	0.0095±0.0138	0.0119±0.0069	0.0009±0.0017
Coalescent depth	0.0089±0.0090	0.0142±0.0196	0.0148±0.0069	0.0012±0.0023

ITS2: Internal transcribed spacer 2

Table 4: Wilcoxon signed rank test for interspecific variations

W+	W-	Inter relative ranks, n, P value	Result
<i>matK</i>	<i>psbA-trnH</i>	W+= 306877.0, W-= 271473.0, n = 1075, P = 0.0821	P>0.05, <i>matK</i> = <i>psbA-trnH</i>
<i>matK</i>	ITS2	W+= 21701.0, W-= 556649.0, n = 1075, P = 4.2474E-152	P<10-10, <i>matK</i> >>ITS2
<i>matK</i>	<i>rbcL</i>	W+= 575250.0, W-= 3100.00, n = 1075, P = 1.0756E-173	P<10-10, <i>matK</i> >> <i>rbcL</i>
<i>psbA-trnH</i>	ITS2	W+= 21847.0, W-= 554354.0, n = 1073, P = 1.4515E-151	P<10-10, <i>psbA-trnH</i> >>ITS2
<i>psbA-trnH</i>	<i>rbcL</i>	W+= 549304.0, W-= 19407.0, n = 1066, P = 4.8884E-153	P<10-10, <i>psbA-trnH</i> >> <i>rbcL</i>
ITS2	<i>rbcL</i>	W+= 573079.0, W-= 977.0, n = 1071, P = 1.3494E-175	P<10-10, ITS2> > <i>rbcL</i>

Table 5: Wilcoxon signed rank test for intraspecific variations

W+	W-	Intra relative ranks, n, P value	Result
<i>matK</i>	<i>psbA-trnH</i>	W+= 1225.0, W-= 206.0, n = 53, P = 6.4680E-6	P<0.05, <i>matK</i> > <i>psbA-trnH</i>
<i>matK</i>	ITS2	W+= 735.0, W-= 696.0, n = 53, P = 0.8629	P>0.05, <i>matK</i> =ITS2
<i>matK</i>	<i>rbcL</i>	W+= 1431.0, W-= 0.0, n = 53, P = 2.3865E-10	P<0.05, <i>matK</i> > <i>rbcL</i>
<i>psbA-trnH</i>	ITS2	W+= 254.0, W-= 827.0, n = 46, P = 0.0017	P<0.05, <i>psbA-trnH</i> >ITS2
<i>psbA-trnH</i>	<i>rbcL</i>	W+= 865.0, W-= 38.0, n = 42, P = 2.3263E-7	P<0.05, <i>psbA-trnH</i> > <i>rbcL</i>
ITS2	<i>rbcL</i>	W+= 521.0, W-= 7.0, n = 32, P = 1.5153E-6	P<0.05, ITS2> <i>rbcL</i>

ITS2: Internal transcribed spacer 2

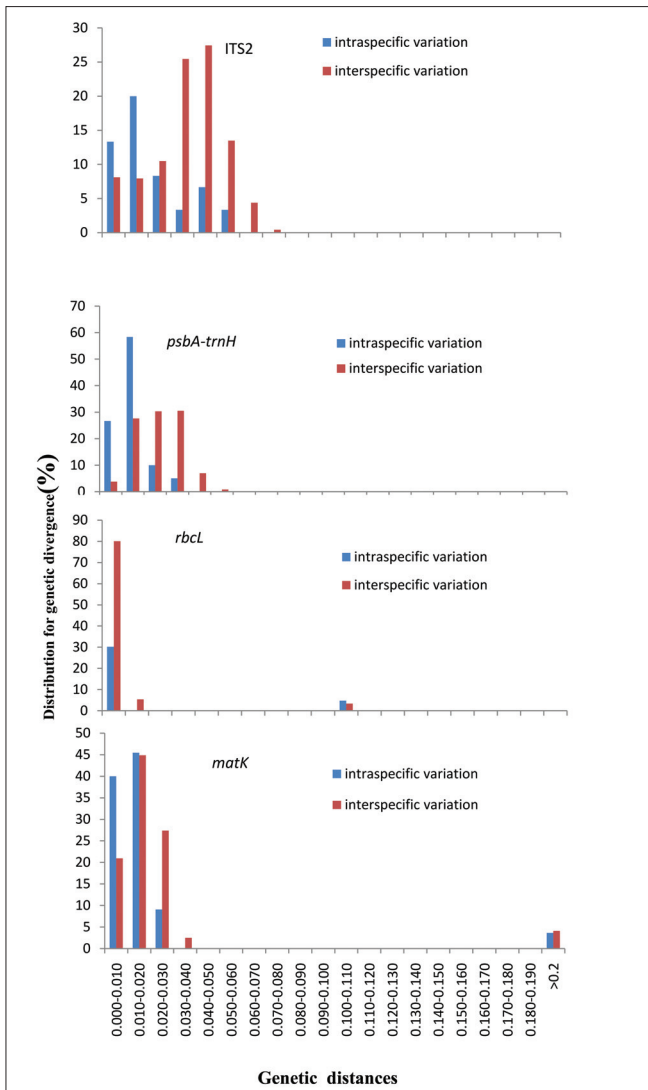


Figure 1: The barcoding gap between interspecific and intraspecific divergences for four candidate barcodes. Histograms showing the relative distribution of pairwise (y-axis) intraspecific (blue bar) and interspecific (red bar) divergence distance estimates (x-axis) for internal transcribed spacer 2 (ITS2), *psbA-trnH*, *rbcL* and *matK* intergenic spacers, respectively. The divergences were calculated using the Kimura 2-parameter model. Barcoding gaps were assessed by the Wilcoxon two-sample tests, and all were significant ($P < 0.05$)

Yao *et al.* found it universal with high success rates of amplification, which is highly recommended in barcode research^[23,24] In our study, we found *psbA-trnH* sequence has a successful identification rate of 70.4%. Although there is a significant difference at intra- and inter-species levels, it has low identification efficiency. Therefore, it is not suitable as the *Ardisia* barcode sequence.

Many researchers have proposed the ITS2 region as a suitable marker for taxonomic classification.^[13,25] However in our study, the identification efficiency with ITS2 is only 51.9%. So ITS2 is also not suitable as a barcode for the identification of *Ardisia* species.

RbcL and *matK* are recommended as plant barcode sequence in the latest Consortium for the Barcode of Life [CBOL] Research.^[26] There are large amount of data for *rbcL* in Genbank, which is universal, being easily amplified and compared, but its variance mainly exist in intra-species rather than inter-species.^[17,27] As described before, *rbcL* fragment was chosen as plant barcode candidate by Kress *et al.*^[8] However, there is no significant difference between the intra-species and inter-species in the research, moreover, the efficiencies of identification by BLAST1 and nearest distance are only 29.1% respectively, therefore, *rbcL* is not proper as the *Ardisia* DNA barcode sequence.

The *matK* fragment is emerging as a gene with potential contribution to plant molecular systematic and evolution.^[28-31]

Table 6: Wilcoxon two-sample tests for distribution of intra- versus inter-specific divergences

Marker	Number of inter-specific distances	Number of intra-specific distances	Wilcoxon	P value
ITS2	1371.0	60.0	72.1	7.18×10^{-4}
<i>psbA-trnH</i>	1371.0	60.0	86.9	3.12×10^{-3}
<i>matK</i>	1075.0	53.0	103.6	5.64×10^{-3}
<i>rbcL</i>	1371.0	60.0	75.1	7.16×10^{-4}

ITS2: Internal transcribed spacer 2

Table 7: Comparison of identification efficiency for potential DNA barcodes loci using different methods of species identification

Marker	Method of species identification	Number of species	Number of samples	Correct identification	Incorrect identification	Ambiguous identification
				Species level %	Species level	Species level %
<i>psbA-trnH</i>	BLAST 1	24	54	70.4	0	29.6
	Distance	24	54	44.4	0	55.6
ITS2	BLAST 1	24	54	51.9	0	48.1
	Distance	24	54	51.9	0	48.1
<i>matK</i>	BLAST 1	22	48	98.1	0	1.9
	Distance	22	48	91.7	0	8.3
<i>rbcL</i>	BLAST 1	24	54	27.8	0	72.2
	Distance	24	54	27.8	0	72.2

BLAST: Basic local alignment search tool; ITS2: Internal transcribed spacer 2; DNA: Deoxyribonucleic acid

The fragment has a quicker evolution compared with other fragments. In the research, the *matK* region had the highest identification success rate at the species level; meanwhile, it exhibited well in PCR amplification and sequencing efficiency, differential intra- and inter-specific divergences and DNA barcoding gap. Therefore, we suggested *matK* region as the DNA barcode for the genus *Ardisia*.

Discussion on samples with unsuccessful identification

In our study, the *matK* sequence was chosen as a DNA barcode to identify the species of *Ardisia* genus. Among the 48 samples, which were successfully sequenced, there was one sample (*A. japonica*) that could not be distinguished from *A. pusilla*. These two species are sister species both attached to the group of Sect. *Bladhia*. They show little differences in morphology and closely relationship between them and that may be the reason that they were difficult to differentiate from each other.

The present research found that of the four candidate loci (*psbA-trnH*, ITS2, *matK*, *rbcL*), *matK* produced the highest rate of successful identification in 91.7% at the species level and it can correctly discriminate 22 Chinese medicinal species from *Ardisia* according to the nearest distance method. Therefore, it is proposed that the *matK* region can be used as a DNA barcode to identify these medicinal plants from *Ardisia*. Collection of more samples and deep researches for those species of ambiguous identification are necessary to provide more effective information about phyletic evolution and more reliable method for the identification of genus *Ardisia*.

Measuring the success rates of identification methods

CBOL recommended *rbcL* and *matK* together as plant barcode sequence, but needs enough matching data from the experiment, which could increase cost, therefore, we just focus on the probability of single sequence, BLAST1/and the nearest distance methods are employed. Meanwhile, identification efficiency was measured in order to display the ability for all sequences. BLAST method compares sample's DNA sequence with total sequence in terms of base, which was ranked by base difference; the advantage of this method is high velocity and accuracy. Nearest distance method compares sample's DNA with the "Kimura 2-parameter (K2P)" distance of total sequence, which is based on overall comparison.^[19] It can quantitate difference in single sequence with low velocity, meanwhile lost locus and variable locus are processed equally, which easily leads to the slight difference between data and facts, which is the reason why these two verifications are not uniform.

We will measure how the total data change when each sequence exist or not during the process of efficiency

identification. When the data are abnormal, we blast the suspicious sequence with GenBank in order to exclude "false positive" data. The same as other authors, we define "inter-species variance" as the variance among different species under a genus without breaking the genus. It might get smaller results than real fact by using "inter-species" in above extent. Layhaye et al.^[17] also got the same conclusion as we have, therefore, we will use some new method of identification, e.g., probability of correct identification [PCI], in order to exclude man-made disturbance.

DNA barcode technique has already been used in animal research and increasing used in plant research, which will assist non-systematic scholars to quickly and accurately identify different species. DNA barcode cannot replace traditional taxonomy, but it is accurate, abundant and unique with high repeats as digital DNA sequence, leading to a useful tool for taxonomists.^[26,27,32] This research explored the application of DNA barcode technique and provided a new method and insight for molecular identification and relationship. As limits of sampling condition in this research, some species had no duplicates; some nearest sibling species were included under a genus. There should be more effective information and reliable method when more samples are included in further research in the future.

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