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Full Length Article

Developing DNA Barcodes for Species Identification of *Berberis* and *Dysosma* Genera in Vietnam

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Abstract

Berberidaceae is the well-known traditional herbal medicine family, including 14 genera and about 700 species, and is considered as a very complex family in term of classification. Nowaday, DNA barcoding can be used to overcome the existed limitation in morphological-based species identification of this family. Therefore, the objective of this study was to find the most efficient DNA barcode regions for identifying species of *Berberis* and *Dysosma* genera. Leaf specimens of *Berberis wallichianae*, *Berberis julianae*, *Mahonia bealei*, and *Dysosma difformis* were collected and dried in silica gel. Total DNA extraction and purification, PCR amplification and DNA sequencing were performed with standard chemicals and kits. Six candidate DNA barcode regions including ITS, *mat*K, 18S, *psb*A-*trn*H, *rbc*L, and *trn*L were amplified, and sequenced. Data were analysed using Seqscape 2.6, DNASTAR Lasergene 7.1, Bioedit 7.0.9.0, and MEGA 7 softwares. Analysis of six obtained DNA barcode sequences of collected samples revealed that ITS and *psb*A-*trn*H regions provided the lowest similarity in sequence and the highest species discriminating power for *Berberis* genus, while ITS and 18S regions were suitable for species identification of *Dysosma* genus. Accordingly, phylogenetic trees based on ITS, *psb*A-*trn*H, and 18S sequences were constructed. In this study, ITS and *psb*A-*trn*H regions were the most efficient markers for molecular identification of species belong to *Berberis* genus. ITS and 18S regions were considered as DNA barcodes for species discrimination of *Dysosma* genus. Our results support the identification of valuable medicinal plant species within *Berberis* and *Dysosma* genera. © 2018 Friends Science Publishers

Keywords: *Berberis*; *Dysosma*; ITS; Molecular marker; *psb*A-*trn*H; Species discrimination

Introduction

Berberidaceae family is one of precious traditional herbal medicines since it contains the alkaloid, berberine, a well-known therapeutic potential sustances for antimicrobial, antioxidant, anti-malaria, and anti-inflammatory (Soffar *et al.*, 2001; Semwal *et al.*, 2009; Singh and Kakkar, 2009; Koncic *et al.*, 2010; Tiwari and Khosa, 2010). Based on mophorlogical data, the family can be classified into 14 genera and about 700 species (Christenhusz and Byng, 2016). However, due to variable morphological characters Berberidaceae is considered a very complex family and its classification solely based on morphology is assumed imprecisely for discrimination at genus level. Therefore, identifying species of Berberidaceae family, particularly Vietnamese distributed *Berberis* and *Dysosma* genera for conservation and exploitation are notified necessarily.

Recently, molecular approach can be used as an effective support to overcome limitation of morphological-based species identification. Among of these molecular techniques, DNA barcoding, is considered as the efficient and accurate tool for global species identification (Chase *et al.*, 2007). The method bases on certain DNA sequence regions of genome, including mitochondrial in animal or chloroplast in plant which has adequately high evolutional rate enough to discriminate species in the same genus (Hebert *et al.*, 2003). Almost all DNA barcodes utilized for classification in plants belong ITS regions in nuclear genome, and *trn*H-*psb*A (Kress *et al.*, 2005), *mat*K, *rbc*L (CBOL Plant Working Group, 2009), *rpo*C1, *rpo*B (Chase *et al.*, 2007) regions in chloroplast genome. The *mat*K (maturase) gene is about 1500 bp long, encodes group II intron maturase and responses for post-transcription process of plant such as photosynthesis (Selvaraj *et al.*, 2008). This gene is moderately variable at genus level, therefore, becomes a potential candidate in evolutionary and botanic classification studies (Hilu and Liang, 1997; Lahaye *et al.*, 2008). The *rbc*L gene encodes a large subunit of ribulose 1, 5-bisphosphate carboxylase/oxygenase (RuBisCO), a key enzyme involved in the first step of Calvin cycle in C3

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plants. Approximate 1400 bp in length, it contains an open reading frame (ORF) and has low rate of insertions and deletions. Nowadays, *rbc*L becomes one of the most frequently segments and widely used in phylogenetic analysis and botanical taxonomy (Chase *et al.*, 2007). Another DNA barcode in chloroplast genome is an intergenic spacer region located between *psb*A and *trn*H gene in chloroplast DNA (Pang *et al.*, 2012). It is about 600 bp in length and its highly variable change region is considered as a potential candidate for a standard DNA barcode. In addition to the molecular markers in chloroplast genome, the spacers between ribosome coding genes in nuclear genome called Internal Transcribed Spacers (ITS) are described as ITS1 (located between 18S and 5.8S) and ITS2 (located between 5.8S and 28S), evolve quickly and vary considerably, thus suitable for use as standard DNA barcodes (Liu *et al.*, 2009; Tripathi *et al.*, 2013; Keskin *et al*., 2017). Besides, several studies demonstrated that in plant two barcodes usually acquire discrimination better than a single marker. Therefore, due to the straightforward recovery of the *rbc*L region and the discriminatory ability of *mat*K region, *rbc*L+*mat*K combination usually provide the best results (Chase *et al.*, 1993; Hilu and Liang, 1997; CBOL Plant Working Group, 2009). However, Pang *et al.* (2012) reported that *psb*A-*trn*H+ITS2 outperformed in both direct sequencing and species identification, compare to other combinations in most taxonomic level. In 2015, Huang reported that ITS outperformed, *psb*A-*trn*H was slightly less effective while *rbc*L and *mat*K gave poor results in species and genus identification (Huang *et al.*, 2015). The combination of the two markers, ITS and *mat*K was reported to result in higher rates of species determination and genetic diversity estimation of *Ancistrocladus* genus (Lien *et al*., 2017).

In recent years, DNA molecular marker and DNA barcode techniques have been applied to study genetic relationship and classification of Berberidaceae species. Kim *et al*. (2004) had buid a phylogenetic tree of Berberidaceae family based on *ndh*F gene. The number of variable regions of *ndh*F gene is known as twice as *rbc*L. In 2010, Roy's study assumed that ITS, *psb*A-*trn*H, *mat*K and *rbc*L did not work in complex groups such as Indian *Berberis* species (Roy *et al.*, 2010). Research on the genus *Dysosma* of Gong *et al.* (2006) showed that two chloroplast intergenic regions *trn*T-*trn*L and *trn*D-*trn*T could be used for the identification of *Dysosma* species.

As the importance and challenge in classification of Berberidaceae family as well as *Berberis* genus, in this study, the identification of four species *Berberis wallichianae, Berberis julianae, Mahonia bealei* and *Dysosma difformis* collected in Vietnam were investigated based on the analysis of *psb*A-*trn*H, ITS, *rbc*L, matK, 18S, and *trn*L regions from three individuals per species. The obtained results would contribute an additional DNA database for specifically identifying species in Berberidaceae family.

Materials and Methods

Materials

Leaves of *B. julianae, M. bealei* and *D. difformis* were collected from Hoang Lien Son National Park of Lao Cai province and Mai Chau of Hoa Binh province by Vietnam National University of Forestry. Leaves of *B. wallichianae* were collected from Sa Pa of Lao Cai province by the Institute of Regional Research and Development,Vietnam Ministry of Science and Technology (Fig. 1). This research was conducted at the Institute of Genome Research, Vietnam Academy of Science and Technology from April, 2016 to March, 2017.

Methods

Total DNA extraction and purification: Leaf specimens of three individuals per species named BJ01-03, MB01-03, DD01-03, and BW01-03 were used for total DNA extraction following the protocol described by Iqbal *et al.* (2013) with slight modifications of sample's weight and grinding step to make it suitable for plant specific tissue and storage conditions. Briefly, 50 mg of leaf tissues from each individual was well-grounded in liquid nitrogen. Cell wall and membrane were degraded by suspending in a buffer composed of 1.4 M NaCl (Scharlau, Spain), 0.1 M Tris-HCl (Bio Basic, Canada) pH 8.0, 20 mM EDTA (Merck, Germany) pH 8.0, 2% CTAB (Affymetrix, USA), 1% PVP (Sigma-Aldrich, USA), and 0.1% β-mercaptoethanol (Sigma-Aldrich, USA) for 35 min at 65^{o}C. The treatments were then hold at room temperature within 5 min. Cell fragments and pellets were removed by adding one volume of chloroform:isoamyl alcohol (Merck, Germany) (C:I, 24:1, v:v) and centrifuged at 6000 rpm for 15 min at 4^{o}C. The supernatants were transferred to new test tubes, added 1 µL RNAse (Thermo Fisher Scientific, USA) and incubated for 15 min at 37^{o}C. The supernatants were purified by adding C:I (24:1, v:v), centrifuging at 6000 rpm for 15 min at 4^{o}C, transferring to clean eppendorf tubes, and adding half volume of NaCl 5 M. DNA was then precipitated by two volume of absolute ethanol for 20 min at 4^{o}C, centrifuged at 3000 rpm for 3 min, and at 8000 rpm for 5 min at 4^{o}C. The precipitated DNA was washed by 70% ethanol and dried. Finally, the DNA was resuspended in sterilized deionized water.

Amplification of ITS, *mat*K, 18S, *psb*A-*trn*H, *rbc*L, and *trn*L Regions and PCR Product Purification

Primers for PCR of these specific DNA regions were designed based on the GenBank sequences as shown in Table 1. Each target DNA region was amplified in a PCR volume of 20 µL containing 1X DreamTaq buffer, 200 mM of each dNTP, 2.5 µM of each primer, 1.5 mM $MgCl_2$, 0.75 units of Dream Taq DNA polymerase (ThermoFisher Scientific, USA) and 50 ng of template DNA.

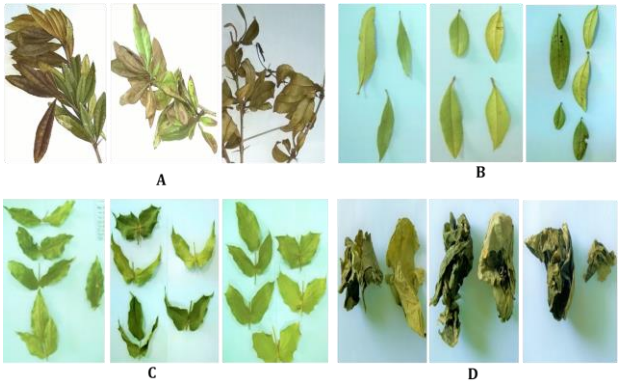

Fig. 1: Plant samples used for molecular study. (A) *Berberis wallichiana* dc, BM01, BM02, BM03. (B) *Berberis julianae* C.K.Schneid, BJ01, BJ02, BJ03. (C) *Mahonia bealei* (Fortune) Pynaert, MB01, MB02, MB03. (D) *Dysosma difformis* T.H.Wang, DD01, DD02, DD03

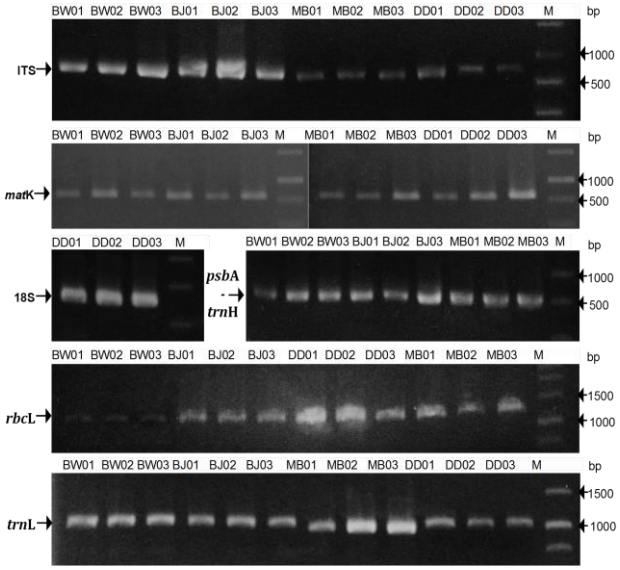

Fig. 2: Electrophoresis of PCR products of amplified target genes

The PCR was performed by IBM Veriti (Applied Biosystems, USA) for 2 min at 94°C denaturation, 35 amplification cycles (30 s at 94°C denaturation, 20 s at 50–55°C annealing, and 1 min at 72°C extension), 5 min at 72°C extension, then hold at 4°C. The amplified products were purified using GeneJET™ PCR Purification Kit (ThermoFisher Scientific, USA) as described by the manufacturer, then screened on 1% agarose gel.

Sequence Analysis and Alignment

Purified DNA was sequenced by ABI 3500 Genetic Analyzer applying BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, USA). The sequencing mixture was prepared in total volume of 15 µL containing 1X BigDye buffer, BigDye, 3.2 pM primer, and 200 ng purified DNA. The PCR was performed by IBM Veriti (Applied Biosystems, USA) for 1 min at 96°C denaturation, 25 amplification cycles (10 s at 96°C, 5 s at 50°C, and 4 min at 60°C), then hold at 4°C. After amplification, PCR product was purified by ethanol/EDTA method. Briefly, 5 µL of 125 mM EDTA and 60 µL of absolute ethanol were added to the tube of PCR product and kept at room temperature for 15 min. The supernatant was discarded after centrifuging at 12000 rpm for 15 min. Next, 60 µL of 70% ethanol was added and centrifuged again at 10000 rpm for 10 min. The acquired pellet contained precipitated DNA was dried. The purified DNA was then denatured by adding 10 µL of Hi-Di Formamide at 95°C for 5 min. The samples were loaded to each well of a sample tray, and performed electrophoresis in 80 cm × 50 µm capillary tubes with POP-4 polymer (ABI, USA). The nucleotide sequence of each sample was identified both forward and reverse directions.

All raw obtained sequences were assembled and analysed using Seqscape 2.6 (Applied Biosystems, USA), DNASTAR Lasergene 7.1 (DNASTAR, USA), Bioedit 7.0.9.0 softwares (Hall, 1999). Pairwise distance was performed using MEGA 7 (Kumar *et al.*, 2016). The phylogenetic trees were constructed using Neighbor Joining and Maximum Likelihood methods with bootstrap value equal 1000.

Results

Efficiency of PCR Amplification of Target DNA Regions

Total DNA of all samples were extracted and used as templates for amplifying target DNA regions. Amplicons acquired with specific primers for ITS, *mat*K, 18S, *psb*A-*trn*H, *rbc*L, and *trn*L regions represented precise lengths as expected (850 bp, 770 bp, 400 bp, 580 bp, 1270 bp and 1038 bp, respectively). The success rates of PCR amplification were high for all regions (Fig. 2).

Assessment of Barcoding Gap and Authentication Ability

To estimate the identification ability using DNA barcoding, BLAST tool was used for determining the identity of a sample based on the best hit of the query sequence, and the E-value for the match must be less than the cut-off value. Accordingly, all published sequences of interestregions of genera *Berberis*, *Dysosma* and *Mahonia* were retrieved to construct a reference sequence library for alignment with obtained sequences in this study.

The results showed that these target DNA regions were successfully sequenced. In this analysis, *Mahonia* and *Berberis* were combined as one genus since recent publications of taxonomy did not separate species of the two genera due to the lack of reference sequences (Yu and Chung, 2014). Within the genus *Berberis*, the percentage of

Table 1: Sequences of primers utilized for amplifying ITS, matK, 18S, psbA-trnH, and trnL regions

DNA region	Primer	Sequence (5'-3')	Length of PCR product (bp)
ITS	ITS–AB101	ACG AAT TCA TGG TCC GGT GAA GTG TTC G	850
	ITS–AB102	TAG AAT TCC CCG GTT CGC TCG CCG TTA C	
*mat*K	Mt–*mat*K–F	ACC CCA TCC ATC TGG AAC	768
	Mt–matK–R	CAA ATC TAT CGA TAA TAT CAG AAT CCG	
18S	Ds-18S-F	GTG CAG CGA ACC CCA TAC	400
	Ds-18S-R	AGC ATT CCT TCA ACA CAC C	
*psb*A-*trn*H	Be–*psb*A–*trn*H–F	CTA GAT TTA GCT GCT GTT GAA GC	580
	Be–psbA–trnH–R	ATT CAC AAT CCA CTG CCT TAA CCC	
*rbc*L	Mt–*rbc*L–F	GAT ACT GAT ATC TTG GCA GCA TTC C	1270
	Mt–rbcL–R	GCT AGT TCA GGG CTC CAT TTG	
*trn*L*	*trn*Lf	ATT TGA ACT GGT GAC ACG AG	1038
	trnLc	CGA AAT CGG TAG ACG CTA CG	

* Primers of this region were synthesized following Taberlet *et al.* (1991)

Table 2: Proportion of similarity between DNA barcode sequences of species in genera *Berberis*, *Dysosma*, *Mahonia*, *Disporopsis* and reference database from GenBank

Table 2A: ITS region of samples BW01, BW02, BW03, BJ01, BJ02, BJ03, MB01, MB02, MB03

Reference species	ITS								
	BW01	BW02	BW03	BJ01	BJ02	BJ03	MB01	MB02	MB03
Berberis wallichiana	100.00	100.00	100.00	100.00	100.00	100.00	97.55	97.55	97.55
Berberis julianae	99.62	99.62	99.62	99.62	99.62	99.62	97.36	97.36	97.36
Berberis koreana	98.87	98.87	98.87	98.87	98.87	98.87	97.55	97.55	97.55
Berberis amurensis	98.87	98.87	98.87	98.87	98.87	98.87	98.30	98.30	98.30
Berberis bealei	96.98	96.98	96.98	96.98	96.98	96.98	98.68	98.68	98.68
Berberis aquifolium	96.79	96.79	96.79	96.79	96.79	96.79	96.98	96.98	96.98
Berberis gracilipes	96.98	96.98	96.98	96.98	96.98	96.98	97.36	97.36	97.36
Berberis ilicifolia	98.12	98.12	98.12	98.12	98.12	98.12	96.99	96.99	96.99
Berberis microphylla	98.31	98.31	98.31	98.31	98.31	98.31	97.19	97.19	97.19
Berberis nervosa	97.36	97.36	97.36	97.36	97.36	97.36	97.93	97.93	97.93
Berberis nevinii	97.37	97.37	97.37	97.37	97.37	97.37	97.18	97.18	97.18
Berberis pallida	95.67	95.67	95.67	95.67	95.67	95.67	95.86	95.86	95.86
Berberis sargentiana	100.00	100.00	100.00	100.00	100.00	100.00	97.55	97.55	97.55
Berberis valdiviana	98.12	98.12	98.12	98.12	98.12	98.12	97.19	97.19	97.19
Mahonia fortunei	97.36	97.36	97.36	97.36	97.36	97.36	98.68	98.68	98.68
Mahonia jingxiensis	96.79	96.79	96.79	96.79	96.79	96.79	98.49	98.49	98.49
Average similarity (%)		97.82 (0.09)							

Figure in parenthesis indicates standard deviation

sequence similarity was highest for *mat*K (99.52%), followed by *rbc*L (99.31%), *trn*L (98.07%), and ITS (97.82%). The proportion of *psb*A-*trn*H region was exhibited the lowest with 94.95%. In the genus *Dysosma*, however, the region achieved the highest proportion of similarity was *rbc*L, at 99.53%. Other regions *mat*K, *trn*L, ITS, and 18S showed the lower rates at 99.26%, 97.43%, 97.13% and 95.10%, respectively (Table 2). The high levels of divergence in ITS, and *psb*A-*trn*H were also demonstrated in previous study (Kress *et al.*, 2005).

Using six *rbc*L sequences of BW01-03, BJ01-03 samples as a query yielded the best matches which were either 100% identical to *rbc*L sequences of known species *B. wallichianae* and *B. pruinosa*, or at a 99.91% sequence similarity to those of *B. julianae*, *B. dumicola*, and *B. sargentiana*. For *mat*K region, sequences of these samples were matched 100% identity with *B. dumicola* and showed a high degree of homology (99.86%) to *B. koreana*, *B. amurensis*, *B. kawakamii*, and *B. pruinosa*. When high variable regions such as ITS were investigated, sequence identity of the samples BW01-03 and BJ01-03 were 100% in comparison with *B. wallichiana*, *B. sargentiana* and 99.62% with *B. julianae*. For the highest variable region *psb*A-*trn*H, however, the results of these samples were slightly lower, at 99.58% for *B. wallichiana* and 99.37% for *B. pruinosa*. These results indicated that the samples BW01-03 and BJ01-03 were most closely related to *B. wallichiana* as well as the group *B. julianae*, *B. pruinosa*, *B. sargentiana*, and *B. dumicola*.

The comparison of *rbc*L sequences between three specimens MB01-03 and reference sequences of species within *Berberis* genus demonstrated that MB01-03 showed the highest similarity level at 99.83% to *B. bealei*. In *mat*K region, these samples were 100% identical to *B. bealei*, and 99.86% to *B. gracilipes*. The rate of similarity was marginally lower in the high variable ITS region, particularly with species *M. fortunei* (98.68%) and *M. jingxiensis* (98.49%). For the highest variable region *psbA-trnH*, MB01-03 had the most remarkable similarity to *B. gracilipes* and *B. bealei* (99.79 and 97.73%, respectively).

Table 2B: *mat*K region of samples BW01, BW02, BW03, BJ01, BJ02, BJ03, MB01, MB02, MB03

Reference species	*mat*K								
	BW01	BW02	BW03	BJ01	BJ02	BJ03	MB01	MB02	MB03
Berberis koreana	99.86	99.86	99.86	99.86	99.86	99.86	99.42	99.42	99.42
Berberis amurensis	99.86	99.86	99.86	99.86	99.86	99.86	99.42	99.42	99.42
Berberis bealei	99.28	99.28	99.28	99.28	99.28	99.28	100.00	100.00	100.00
Berberis thunbergii	98.99	98.99	98.99	98.99	98.99	98.99	99.13	99.13	99.13
Berberis vulgaris	99.71	99.71	99.71	99.71	99.71	99.71	99.28	99.28	99.28
Berberis aquifolium	99.28	99.28	99.28	99.28	99.28	99.28	99.42	99.42	99.42
Berberis dumicola	100.00	100.00	100.00	100.00	100.00	100.00	99.28	99.28	99.28
Berberis gracilipes	99.14	99.14	99.14	99.14	99.14	99.14	99.86	99.86	99.86
Berberis ilicifolia	99.28	99.28	99.28	99.28	99.28	99.28	99.42	99.42	99.42
Berberis kawakamii	99.86	99.86	99.86	99.86	99.86	99.86	99.42	99.42	99.42
Berberis microphylla	99.42	99.42	99.42	99.42	99.42	99.42	99.57	99.57	99.57
Berberis nervosa	99.57	99.57	99.57	99.57	99.57	99.57	99.71	99.71	99.71
Berberis nevinii	99.42	99.42	99.42	99.42	99.42	99.42	99.57	99.57	99.57
Berberis pallida	99.57	99.57	99.57	99.57	99.57	99.57	99.71	99.71	99.71
Berberis pruinosa	99.86	99.86	99.86	99.86	99.86	99.86	99.42	99.42	99.42
Berberis valdiviana	99.42	99.42	99.42	99.42	99.42	99.42	99.57	99.57	99.57
Mahonia japonica	99.28	99.28	99.28	99.28	99.28	99.28	99.71	99.71	99.71
Average similarity (%)		99.52 (0.02)							

Figure in parenthesis indicates standard deviation

Table 2C: *psb*A-*trn*H region of samples BW01,BW02, BW03, BJ01, BJ02, BJ03, MB01, MB02, MB03

Reference species	*psb*A-*trn*H								
	BW01	BW02	BW03	BJ01	BJ02	BJ03	MB01	MB02	MB03
Berberis wallichiana	99.58	99.58	99.58	99.58	99.58	99.58	93.09	93.09	93.09
Berberis julianae	97.95	97.95	97.95	97.95	97.95	97.95	91.24	91.24	91.24
Berberis koreana	94.65	94.65	94.65	94.65	94.65	94.65	93.29	93.29	93.29
Berberis amurensis	94.65	94.65	94.65	94.65	94.65	94.65	91.95	91.95	91.95
Berberis bealei	94.24	94.24	94.24	94.24	94.24	94.24	97.73	97.73	97.73
Berberis thunbergii	96.45	96.45	96.45	96.45	96.45	96.45	94.27	94.27	94.27
Berberis vulgaris	94.65	94.65	94.65	94.65	94.65	94.65	93.09	93.09	93.09
Berberis aquifolium	93.83	93.83	93.83	93.83	93.83	93.83	93.50	93.50	93.50
Berberis dumicola	98.76	98.76	98.76	98.76	98.76	98.76	94.32	94.32	94.32
Berberis gracilipes	93.10	93.10	93.10	93.10	93.10	93.10	99.79	99.79	99.79
Berberis ilicifolia	95.82	95.82	95.82	95.82	95.82	95.82	95.26	95.26	95.26
Berberis kawakamii	97.54	97.54	97.54	97.54	97.54	97.54	91.22	91.22	91.22
Berberis microphylla	95.19	95.19	95.19	95.19	95.19	95.19	95.45	95.45	95.45
Berberis nervosa	87.89	87.89	87.89	87.89	87.89	87.89	88.03	88.03	88.03
Berberis nevinii	94.44	94.44	94.44	94.44	94.44	94.44	93.90	93.90	93.90
Berberis pallid	94.46	94.46	94.46	94.46	94.46	94.46	94.11	94.11	94.11
Berberis pruinosa	99.37	99.37	99.37	99.37	99.37	99.37	93.09	93.09	93.09
Berberis sargentiana	97.95	97.95	97.95	97.95	97.95	97.95	91.24	91.24	91.24
Berberis valdiviana	95.61	95.61	95.61	95.61	95.61	95.61	95.45	95.45	95.45
Average similarity (%)		94.95 (0.21)							

Figure in parenthesis indicates standard deviation

Table 2D: *rbc*L region of samples BW01, BW02, BW03, BJ01, BJ02, BJ03, MB01, MB02, MB03

Reference species	*rbc*L								
	BW01	BW02	BW03	BJ01	BJ02	BJ03	MB01	MB02	MB03
Berberis wallichiana	100.00	100.00	100.00	100.00	100.00	100.00	99.22	99.22	99.22
Berberis julianae	99.91	99.91	99.91	99.91	99.91	99.91	99.31	99.31	99.31
Berberis koreana	99.66	99.66	99.66	99.66	99.66	99.66	99.40	99.40	99.40
Berberis amurensis	99.66	99.66	99.66	99.66	99.66	99.66	99.40	99.40	99.40
Berberis bealei	99.40	99.40	99.40	99.40	99.40	99.40	99.83	99.83	99.83
Berberis thunbergii	99.14	99.14	99.14	99.14	99.14	99.14	98.88	98.88	98.88
Berberis aquifolium	99.31	99.31	99.31	99.31	99.31	99.31	99.40	99.40	99.40
Berberis dumicola	99.91	99.91	99.91	99.91	99.91	99.91	99.14	99.14	99.14
Berberis kawakamii	99.66	99.66	99.66	99.66	99.66	99.66	99.05	99.05	99.05
Berberis pruinosa	100.00	100.00	100.00	100.00	100.00	100.00	99.22	99.22	99.22
Berberis sargentiana	99.91	99.91	99.91	99.91	99.91	99.91	99.31	99.31	99.31
Mahonia bealei	97.93	97.93	97.93	97.93	97.93	97.93	95.57	95.57	95.57
Mahonia japonica	99.22	99.22	99.22	99.22	99.22	99.22	98.02	98.02	98.02
Average similarity (%)		99.31 (0.07)							

Figure in parenthesis indicates standard deviation

The comparison of five DNA regions between MB01-03 and reference sequence of *Berberis* and *Mahonia* genera implied that MB01-03 and two species *B. gracilipes, B. bealei* had the close phylogenetic relationship (Table 2).

The *mat*K sequences of the three specimens DD01-03 of the genus *Dysosma* were 100% identical to each other as well as to the species *D. difformis*. Alignment of *rbc*L sequences showed 99.82% of similarity to both *D. difformis* and *D. pleiantha*.

Table 2E: *trn*L region of samples BW01, BW02, BW03, BJ01, BJ02, BJ03, MB01, MB02, MB03

Reference species	*trn*L								
	BW01	BW02	BW03	BJ01	BJ02	BJ03	MB01	MB02	MB03
Berberis koreana	97.94	97.94	97.94	97.83	97.83	97.83	98.14	98.14	98.14
Berberis amurensis	97.83	97.83	97.83	97.73	97.73	97.73	98.14	98.14	98.14
Berberis bealei	98.13	98.13	98.13	98.03	98.03	98.03	99.58	99.58	99.58
Berberis thunbergii	97.71	97.71	97.71	97.61	97.61	97.61	98.12	98.12	98.12
Berberis koreana	97.94	97.94	97.94	97.83	97.83	97.83	98.14	98.14	98.14
Berberis amurensis	97.83	97.83	97.83	97.73	97.73	97.73	98.14	98.14	98.14
Berberis bealei	98.13	98.13	98.13	98.03	98.03	98.03	99.58	99.58	99.58
Berberis thunbergii	97.71	97.71	97.71	97.61	97.61	97.61	98.12	98.12	98.12
Average similarity (%)	98.07 (0.08)								

Figure in parenthesis indicates standard deviation

Table 2F: ITS region of samples DD01, DD02, DD03

Reference species	ITS		
	DD01	DD02	DD03
Dysosma difformis	98.59	98.80	98.80
Dysosma aurantiocaulis	96.99	97.19	97.19
Dysosma delavayi	96.79	96.79	96.79
Dysosma majorensis	96.99	97.19	97.19
Dysosma pleiantha	96.79	96.59	96.59
Dysosma tsayuensis	96.59	96.79	96.79
Dysosma veitchii	96.79	96.79	96.79
Average similarity (%)	97.13 (0.15)		

Figure in parenthesis indicates standard deviation

Table 2G: *mat*K region of samples DD01, DD02, DD03

Reference species	*mat*K		
	DD01	DD02	DD03
Dysosma difformis	100.00	100.00	100.00
Dysosma aurantiocaulis	99.86	99.86	99.86
Dysosma delavayi	98.31	98.31	98.31
Dysosma majorensis	98.73	98.73	98.73
Dysosma pleiantha	99.72	99.72	99.72
Dysosma tsayuensis	99.30	99.30	99.30
Dysosma veitchii	98.31	98.31	98.31
Dysosma versipellis	99.86	99.86	99.86
Average similarity (%)	99.26 (0.14)		

Figure in parenthesis indicates standard deviation

Table 2H: 18S region of samples DD01, DD02, DD03

Reference species	18S		
	DD01	DD02	DD03
Dysosma difformis	97.69	97.69	97.69
Dysosma aurantiocaulis	93.33	93.33	93.33
Dysosma delavayi	94.02	94.02	94.02
Dysosma majorensis	98.27	98.27	98.27
Dysosma pleiantha	94.80	94.80	94.80
Dysosma tsayuensis	92.46	92.46	92.46
Dysosma veitchii	95.10	95.10	95.10
Average similarity (%)	95.10 (0.45)		

Figure in parenthesis indicates standard deviation

The highest proportion of similarity for *trn*L sequences was 97.65% to those of *D. difformis* and *D. versipellis*. The ITS sequences of DD01-03 were 98.59% similar to that of species *D. difformis*. The 18S gene was one of the most variable regions with the average similarity at 95.10% and the highest similarities were 97.69% similar to *D. difformis* and 98.27% to *D. majorensis*. Alignment of all obtained sequences and reference sequences from GenBank showed that DD01-03 had close relationships with *D. difformis* and a group of *D. majorensis*, *D. versipellis*, and *D. pleiantha* (Table 2).

The genetic variation among species of the genus *Berberis* was described through pairwise distance value in Table 3A. The nucleotide diversity indexes of ITS and *psb*A-*trn*H barcodes were significantly higher than those of *rbc*L, *mat*K and *trn*L, with the mean values of 0.022, 0.019, 0.006, 0.005 and 0.004, respectively. The mean pairwise distance among all sequences of *Dysosma* was summarized in Table 3B.

Table 2I: *rbc*L region of samples DD01, DD02, DD03

Reference species	*rbc*L		
	DD01	DD02	DD03
Dysosma difformis	99.82	99.82	99.82
Dysosma aurantiocaulis	99.46	99.46	99.46
Dysosma delavayi	99.28	99.28	99.28
Dysosma majorensis	99.46	99.46	99.46
Dysosma pleiantha	99.82	99.82	99.82
Dysosma tsayuensis	99.46	99.46	99.46
Dysosma veitchii	99.28	99.28	99.28
Dysosma versipellis	99.64	99.64	99.64
Average similarity (%)	99.53 (0.04)		

Figure in parenthesis indicates standard deviation

Table 2J: *trn*L region of samples DD01, DD02, DD03

Reference species	*trn*L		
	DD01	DD02	DD03
Dysosma difformis	97.65	97.65	97.65
Dysosma aurantiocaulis	97.28	97.28	97.28
Dysosma delavayi	97.40	97.40	97.40
Dysosma majorensis	97.53	97.53	97.53
Dysosma pleiantha	97.40	97.40	97.40
Dysosma tsayuensis	97.28	97.28	97.28
Dysosma veitchii	97.28	97.28	97.28
Dysosma versipellis	97.65	97.65	97.65
Average similarity (%)	97.43 (0.03)		

Figure in parenthesis indicates standard deviation

Table 3: Information of DNA barcodes used for *Berberis* genus (including *Mahonia* genus) and *Dysosma* genus in this study

Table 3A: Information of DNA barcodes used for *Berberis* genus

DNA barcodes used for*Berberis* genus	ITS	*mat*K	*psb*A-*trn*H	*rbc*L	*trn*L
Amount of sample	9	9	9	9	9
PCR efficiency (%)	100	100	100	100	100
Sequencingefficiency (%)	100	100	100	100	100
Length of compared sequences (bp)	537	694	521	1160	975
Mean Pairwise Distance	0.022 (0.000-0.051)	0.005 (0.000-0.010)	0.019 (0.000-0.036)	0.006(0.000-0.024)	0.004(0.000-0.008)

Table 3B: Information of DNA barcodes used for *Dysosma* genus

DNA barcodes used for *Dysosma* genus	ITS	*mat*K	18S	*rbc*L	*trn*L
Amount of sample	3	3	3	3	3
PCR efficiency (%)	100	100	100	100	100
Sequencingefficiency (%)	100	100	100	100	100
Length of compared sequences (bp)	498	711	351	553	823
Mean Pairwise Distance	0.026 (0.000-0.050)	0.009 (0.000-0.021)	0.047 (0.000-0.070)	0.004 (0.000-0.007)	0.003 (0.000-0.005)

The 18S gene was achieved the largest average pairwise distance index, at 0.047, in comparison to the rest barcodes (ITS – 0.026, *mat*K – 0.009, *rbc*L – 0.004, and *trn*L – 0.003). Therefore, sequences from ITS and *psb*A-*trn*H could be used to distinguish species of the genus *Berberis* while ITS and 18S regions were recommended for *Dysosma* genus.

Phylogenetic Reconstruction

Sequence analyses of ITS, *mat*K, 18S, *psb*A-*trn*H, *rbc*L, and *trn*L were used for reconstructing phylogenetic tree based on Neighbor Joining and Maximum Likelihood methods. The sequence similarity and phylogenetic relations of species in the genus *Berberis* (including *Mahonia*) based on five regions (ITS, *mat*K, *psb*A-*trn*H, *rbc*L and *trn*L) determined potential DNA barcodes for molecular identification. The results indicated that the ITS and *psb*A-*trn*H sequences revealed the lowest similarity which meant the nucleotide variation and species discriminating power were the most noteworthy (Fig. 3). Other regions did not show high variation for classifying interested species. Similarily, ITS and 18S were combined markers for identification of species within the genus *Dysosma* (Fig. 3).

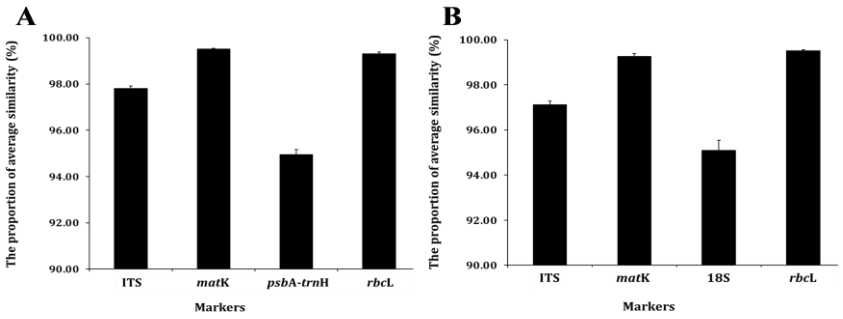

Fig. 3: The average similarity DNA barcode sequences when compare sequences of samples BW, BJ, and MB with *Berberis* genus (A) and samples DD with *Dysosma* genus (B) from GenBank. Average similarities are presented as means ± SD

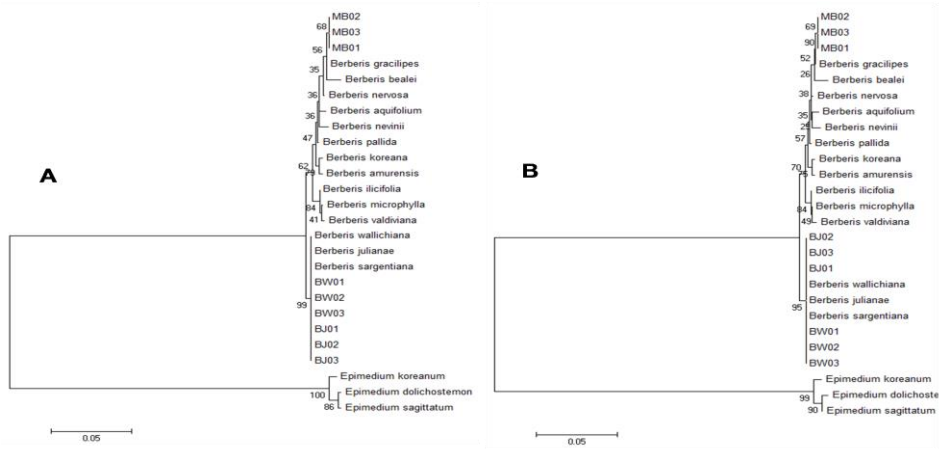

Fig. 4: Phylogenetic tree based on ITS and *psb*A-*trn*H sequences of the *Berberis* genus imaged by Maximum Likelihood (A) and Neighbor Joining (B) methods. Bootstrap values are indicated below the node

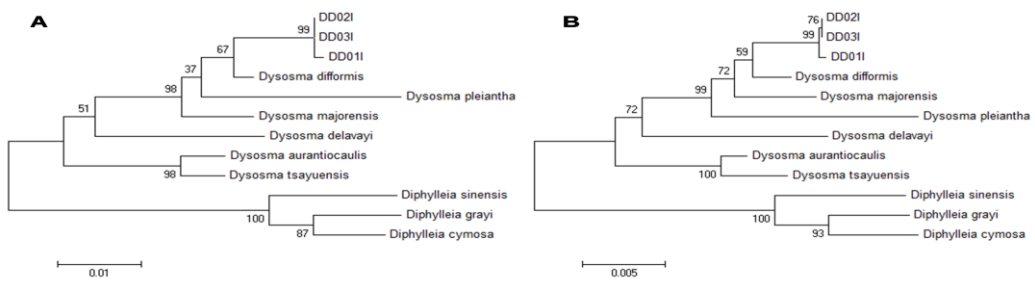

Fig. 5: Phylogenetic tree based on ITS and 18S sequences of the *Dysosma* genus imaged by Maximum Likelihood (A) and Neighbor Joining (B) methods. Bootstrap values are indicated below the node

The phylogenetic trees reconstructed based on two highest variable regions ITS and *psb*A-*trn*H of *Berberis* genus, and ITS and 18S of the *Dysosma* genus were imaged evidently (Fig. 4 and 5).

Corresponding to the phylogeny analysis, samples BW01-03 and BJ01-03 obviously revealed the closest relationships to *B. wallichiana*, then *B. sargentiana* and *B. julianae* with the average percentages of similarity were 99.86%, 99.29% and 99.16%, respectively. A relevant bootstrap value at 99 by Maximum Likelihood method indicated that a confidence interval was eligible for genetic correlation of these species (Fig. 4). For samples MB01-03,

B. bealei and *B. gracilipes* were inferred to be the nearest neighbours with the average similarities at 99.29% and 99.00%. However, the bootstrap value of Maximum Likelihood method was only 56 which signified that a confidence interval for genetic correlation of these species was unqualified (Fig. 4).

ITS and 18S were identified as the most quickly variable and polymorphic regions in *Dysosma* genus and could be used as suitable markers for species classification. In this study, samples DD01-03 showed the close relationship to the species *D. differmis* with the average similarity at 98.75% (Table 2) and low bootstrap value at 67 (Fig. 5). It implied that the correlation between DD01-03 and *D. differmis* was low confidence interval. In addition, in the phylogenetic trees, DD01-03 were usually separated into an isolated group. It was properly belonged to one species of *Dysosma* genus that has not been published sequences on GenBank yet or because of distributional influence.

Discussion

A number of studies have been conducted to find suitable DNA markers for species identification. Different regions as well as combination thereof have been examined and proposed as preferred plant barcodes. In this study, selected species of *Berberis* genus could be identified using the combination of *psb*A-*trn*H and ITS, or 18S and ITS regions in the case of the genus *Dysosma*. These findings on ITS regions are congruent with similar previous studies (Kim and Jansen, 1996; Sun *et al.*, 2005; Bottini *et al.*, 2007; Roy *et al.*, 2010; Mao *et al.*, 2014). ITS regions were selected by different research groups as barcodes for species discrimination in the family Berberidaceae, the genera *Berberis* and *Dysosma*. Due to the fast evolutionary rate, ITS sequences have the utility in taxonomy classification at both the infrageneric and intergeneric levels. Mao *et al.* (2014) found that ITS could be used as a single DNA barcode for species separation of Podophylloideae (including *Dysosma* genus) in Berberidaceae family based on the analysis of nine barcode regions (*mat*K, *rbc*L, *atp*H–*atp*I, *rpl32*–*trn*L^{UAG}, *rps*18–*clpp*, *trn*L–*trn*F, *trn*L–*ndh*J, *trn*S–*trnf*M, and ITS). However, ITS region itself could not discriminate all species of the genera *Berberis* and *Dysosma* (Yu and Chung, 2014). In some other reports, *psb*A-*trn*H locus showed better identification ability than ITS region in the family Berberidaceae or the genus *Berberis* in particularly (Jiang *et al.*, 2011; Yu and Chung, 2014). Jiang *et al.* (2011) analysed four DNA regions of *psb*A-*trn*H, ITS, *rbc*L, and *mat*K and reported that *psb*A-*trn*H region was the best potential DNA barcode to determine phylogeny of ten species of *Epimedium* genus due to the highest interspecific divergence and sequencing rate. Roy *et al.* (2010) reported that none of the barcodes (ITS, *trn*H-*psb*A, *rbc*L, or *mat*K) could identify all species of *Berberis* and the combination of ITS and *psb*A-*trn*H provided the highest divergence at interspecific level. The combination of *psb*A-*trn*H and ITS was also proved to have equal or better identification rate in comparison with other locus combination in most taxonomic groups investigated by Pang (Pang *et al.*, 2012). In the genus *Dysosma*, none of the previous studies reported the discrimination ability of 18S region. The current study found that the combination of ITS and 18S regions improved the species identification power in this genus. However, due to the lack of reference sequences of *Berberis*, *Mahonia* and *Dysosma* species, individual DNA region was probably not suitable for species discrimination. Therefore, the combination of multiple DNA barcodes and morphology are extremely recommended.

Conclusion

In this study, the analysis of DNA barcode regions including ITS, *mat*K, 18S, *psb*A-*trn*H, *rbc*L and *trn*L revealed that ITS and *psb*A-*trn*H regions are combined markers for species classification in *Berberis* genus, while ITS and 18S regions are preferred barcodes for the genus *Dysosma*. The species identification utilizing molecular markers for several species of Berberidaceae family can support morphological classification method and generate a foundation for conservation and commercialization of these important herbal medicines in Vietnam.

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