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The Best DNA Barcoding Marker for Classification of Berberidaceae

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Abstract: Berberidaceae contains 17 genera and nearly 650 species, inwhich genus *Berberis* and genus *Mahonia* are sister with very closed relationship and share many similar characteristics. In the past, two genera were merged as genus *Berberis.* At present, Berberidaceae is at risk and prioritized for conservation, especially the species of *Mahonia* and *Berberis*. In the fact, identification for Berberidaceae based on the morphology of parts for sale (such as roots, stems, leaves) without reproductive organs (flowers and fruits) is impossible. In recent years, molecular biology techniques are being applied widely and effectively in research on evolution, classification and genetic diversity of population. Research based on DNA have highly accurate and particularly useful for closely related species which morphological observations are not sufficient to distinguish. Results from DNA analysis allows to authentic species, populations or individuals from un-intact specimens accurately and especially, it is not affected by objective factors such as the environment or human. In this study, we assessed the taxonomy ability of three commonly DNA barcoding regions used for classification including *rbc*L, *trn*H-*psb*A and *ITS2* on 12 samples of the Berberidaceae family, in which 7 samples are genus *Mahonia*, 4 samples are genus *Berberis* and 1 sample of *Epimedium* was used as control samples. The results of the study will contribute to the selection of suitable DNA markers for the classification of *Mahonia* and *Berberis* samples. The gotten results demonstraded that the *rbc*L region showed the most obvious ability to distinguish 12 Berberidaceae species. The *ITS2* sequences of *Berberis julianeae and Mahonia bealei* of Vietnam were submitted into GB with accession number as MT073031 and MT008067, respectively. The sequence of rbcL of *Mahonia bealei* also submitted to Genbank with accession number MT457415.1

Key words: Berberidaceae, ITS2, *rbc*L, *trn*H-*psb*A, selection of candidate DNA markers

1. Introduction

The first person who mention to the taxons of the Berberidaceae is Linnaeus in 1753. Berberidaceae is the well-known traditional herbal medicine family including 17 genera and about 650 species that distributed mainly in the tropical, subtropical and temperate regions. According to Nguyen Tien Ban (2003), in Vietnam, the Berberidaceae family has 4 genera and 9 species, distributed mainly in the highlands of the northern mountainous provinces and Lam Dong province. All Berberidaceae species are valuable medicinal plants with high economic value, but due to over-exploitation and trading, it has declined sharply in both quantity and quality. At present, all the species of Berberidaceae family are listed in the Vietnam Red Book at Endangered Level (EN) and listed to IA group of Cites in Vietnam (the endangered species, prohibited from export, import, re-export, import from the sea and transit of specimens exploited from the wild for commercial purposes). Identifying species from parts of organ (such as stems, roots, or leaves) without reproductive organs (flowers and fruits) based on morphology is impossible.

DNA barcoding is a modern technique that uses short DNA fragments to standardize differentiation between species [1, 2]. They have become a new tool for forensic, classification, evaluation of genetic

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relationships, quality management, origin of the biological products. In flowering plants, some chloroplast genomic regions (such as matK, rbcL, psbA-trnH, atpF-atpH ...) and nuclear genome region (such as ITS-rDNA, 18S ...) are being widely used in research of phylogeny, taxonomy, and species identity [3, 4]. However, for each different family, the classification ability of these gene regions is different. The study of Song Xue et al. (2019) [5] published in the journal Horticulture showed that 3 genomic regions trnC-psbD, ndhD and atpA-atpH are the 3 most effective gene regions to distinguish species in the genus Plum-apricot (Prunus). Muellner et al. (2011) [6] demonstrated that the ITS gene region is most effective for identification of Meliaceae species. Trang et al. (2015) [7] demonstrated that the combination of three gene regions rbcL, matK and trnH-psbA was effective in distinguishing *Hopea* species. Stoeckle et al. (2011) [8] used a combination of rbcL and matK gene regions to identify rare medicinal plants that are heavily traded in North Africa, but their one obstacle is the lack of DNA comparable data.

In this study, we did sequence of *rbc*L, *trn*H-*psb*A and *ITS*2 genes of 12 species of the Berberidaceae family to test the classification ability of three DNA barcoding markers on species of *Mahonia* and *Berberis* (Berberidaceae). This study is also supplemented the DNA database for the international gene bank, contributing to a more complete database for the gene bank of Berberidaceae species distributed in Vietnam.

2. Material and Methods

2.1 Materials

Total 12 leaf samples of 7 species from genus *Mahonia*, 4 species of genus *Berberis* and 1 sample of *Epimedium* were collected by the research team of the Department of Plant Ethnology, Institute of Ecology and Biological Resources during their field trips on 2020 in several provinces at the north and high land of Vietnam. The information of the collected samples is shown in the Table 1.

Table 1 The information of the collected samples.

Order	Sympol	Scientific name	Collected places
1	BHG	*Mahonia* sp4	Ha Giang province
2	BHG	*Mahonia* sp5	Ha Giang province
3	DL 01	*Mahonia klossii*	Đa Lat-Lam dong province
4	DL 02	*Mahonia klossii*	Đa Lat-Lam dong province
5	BHG	*Mahonia* sp3	Ha Giang province
6	BCB	*Mahonia* sp1	Cao Bang province
7	BBK	*Mahonia* sp2	Bac Kạn province
8	SB 01	*Berberis julianea*	Sa Pa- Lao Cai province
9	SB 02	*Berberis julianea*	Sa Pa- Lao Cai province
10	SB 03	*Berberis julianea*	Sa Pa- Lao Cai province
11	SB 04	*Berberis julianea*	Sa Pa- Lao Cai province
12	DDH	*Epimedium* sp	Sample bought from China

2.2 Methods

Total genomic DNA was extracted according the method of Doyle & Doyle (1990) [9] that under local laboratory conditions.

2.3 PCR Amplification

*ITS*2 region with 300 bp, *trn*H-*psb*A with 400 bp and *rbc*L region with 600 bp in length were amplified using universal ITS1/ITS2 primer [10], rbcLF/R and *trn*H-*psb*A [2]. PCR was performed in 25µl of reaction system containing 7 µl deionized H_2O, 12.5 µl of PCR Master mix kit (2X); 1.25 µl of each primer (10

pmol/µl); 3 µl of DNA template (10-20 ng). The PCR reaction was performed using PCR Model 9700 (GeneAmp PCR System 9700, USA) for 3 min at 94°C for denaturation, 35 amplification cycles (45 s at 94°C for denaturation, 30 s at 55°C annealing and 30 s at 72°C for extension), then 10 min at 72°C for extension and hold at 4°C.

2.4 Sequence Analysis and Alignment

PCR product was screened by electrophoresis on 1% agarose gel, then sequenced at FirstBase Co. Ltd. (Malaysia). Raw sequences obtained were assembled and edited by Chromas-Pro 2.1.6 (Technelysium Pty Ltd, Helensvale, Queensland, Australia). All the sequences were then aligned on BLAST, Genbank[1]. Pairwise distance was determined using Mega 7.0 [11]. The phylogenetic trees were constructed using Maximum Likelihood (ML) and Bayesian Inference (BI) with bootstrap value of 1000.

3. Results and Discussion

3.1 Total DNA Extraction

Total DNA of 12 study samples were successfully DNA extracted with radio $OD_{260nm}/OD_{280\ nm}$ of all goten total DNA range from 1.81-1.83.

3.2 Efficiency of ITS-PCR Amplification

Total DNA of all samples were used as templates for amplifying *ITS*2-rDNA (300 bp), *trn*H-*psb*A (400 bp) and *rbc*L (600 bp) regions.

3.3 Sequencing and Analysis

All PCR products after purification are sequenced at Firstbase Co. Ltd. The results of sequencing were then checked for similarity with the available sequences on the Gene Bank (GB) using BLAST tool. Usually, BLAST results do not give exactly conclusions about species, but in cases where BLAST has high coverage and similarity (above 98%) it may

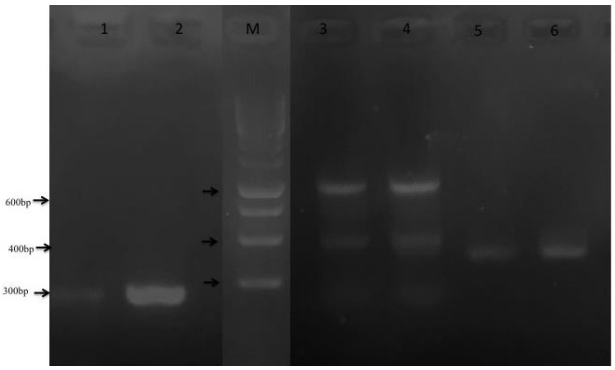


Fig. 1 PCR products of 6 samples representing 3 amplified gene regions tested by electrophoresis on 1% agarose gel.

give a suggestion about the species which has most closely relatonship to the study sample. In this study, the sequences of 3 DNA regions *ITS*2, *rbc*L, *trn*H-*psb*A of all 12 study samples showed high similarity index (over 95%) corresponding to *ITS*2, *rbc*L, *trn*H-*psb*A DNA regions of the *Mahonia* and *Berberis* species on the GB, this demonstrates that the initial morphological classification of the samples is correct, as well as demonstrates that our cloned DNA sequences are successful.

3.4 The Classification Ability of Three DNA Markers rbcL, ITS2 and trnH-psbA on 12 Species of Berberidaceae

The sequence of three DNA regions include *rbc*L, *ITS2* and *trn*H-*psb*A were analyzed by Mega 7.0 soft ware, align and assessment the classification ability of these three DNA regions base on the genetics distance of 12 samples. The genetic relationship diagram of 12 study samples based on sequence of *rbc*L (A), *trn*H-*psb*A (B), *ITS* (C) was shown in the Fig. 2.

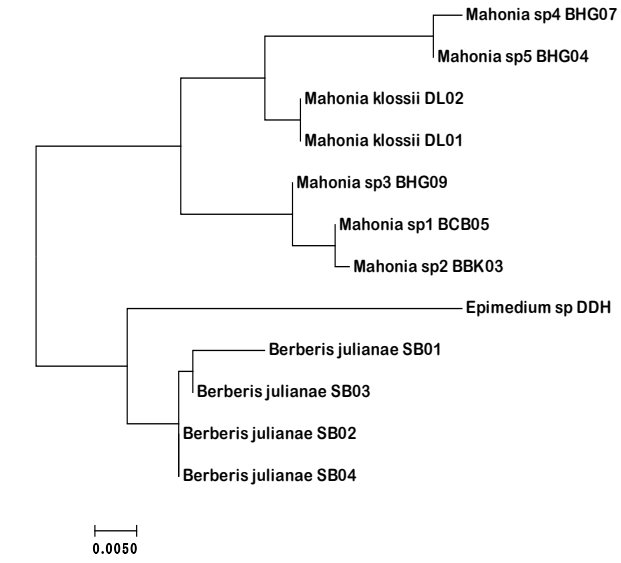
Results of phylogeny based on the method of Maximum Likelihood (ML)) of 12 study samples (Fig. 2) showed that:

When analyse based on the sequence of *rbc*L with 600 bp in length, 12 samples of Berberidaceae were separated into 3 difference branches, 7 samples of *Mahonia* genus separated into 1st branch, 4 samples of *Berberis* genus in the other branch, and *Epimedium*

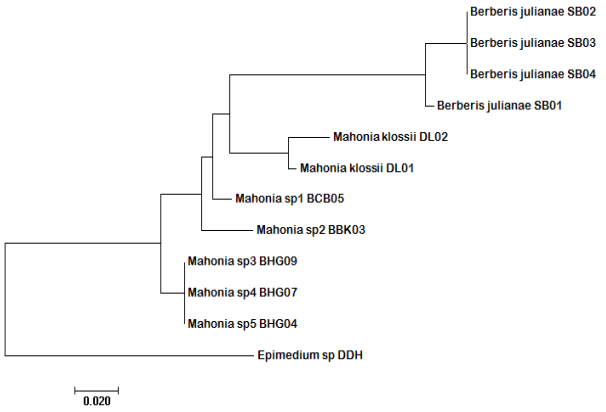
[1] http//:www.ncbi.nlm.nih.gov/BLAST.

sp. in the 3rd clade and closer to the species of the genus *Berberis* than *Mahonia* (Fig. 2a).

When analyse based on the sequence of *trn*H-*psb*A with 400 bp in length, 4 *Berberis* samples were separated into 1 branch, 7 *Mahonia* samples in another branch and the control species *Epimedium* sp. always in an independent branch compared to the two groups of *Berberis* and *Mahonia*. However, 7 *Mahonia* samples continue were divided into 2 groups, where 3 *Mahonia* samples that collected in Ha Giang (BHG 04, BHG 07, BHG 09) concentrated on a small branch, while the remaining 4 *Mahonia* samples separated into other small branch (in which BBK, BCB samples collected in Cao Bang and Bac Kan were separated into 2 different branches, 2 samples DL 01 and DL 02 samples collected in Da Lat (Lam Dong province) were in the same branch. This separation can be considered as an attractive result because in terms of taxonomy at the species level, *trn*H-*psb*A gave correct results when accurately dividing 7 *Mahonia* samples and 4 *Berberis* samples into several smaller branches following exactly their collected places So, it seems that the *trn*H-*psb*A DNA region can be divided the geographically samples. However, to prove this, it is necessary to have a larger number of samples as well as need more samples of the same species collected at different locations for control. In this study, the goal was to evaluate the ability of identification at the species level, so if the *trn*H-*psb*A DNA region is really able to distinguish both species and sub-species (or geographic species) is considered inappropriate because it easily make the confusion leads to false conclusions about the specimen need identified. Therefore, from the perspective of using molecular markers to classify at the species level, the *trn*H-*psb*A gene region would be inconsistent. However, in researching, *trn*H-*psb*A will be a DNA region containing many attractions, because it is seemly that this DNA region will help assess the dissociation capacity of geographic species or sub-species of Berberidaceae.



(a) *rbc*L



(b) *trn*H-*psb*A

(c) ITS2

Fig. 2 (a) The genetic relationship diagram of 12 study samples based on sequence of *rbc*L, (b) *trn*H-*psb*A, (c) *ITS*.

For ITS2-rDNA region: Unlike 2 genomic regions *rbc*L-600 and *trn*H-*psb*A, 12 samples belonging to 3

genera, when analyzed based on ITS2-sequence, they were divided only into 2 main branches, in which the sample of *Epimedium* sp. belong to 1 large branch and the remaining 11 samples are in the second largest branch. The *Mahonia* and *Berberis* samples are located alternately in small branches, not clearly divided into 2 branches as the obtained results when analysed based on the sequences of *rbc*L-600 bp and *trn*H-*psb*A. ITS2 in the nuclear genome is a more stable than the DNA in the chloroplast genome. Normally, for samples that have genetic distance is not large enough, it is difficult to separate if base on sequence of stable nuclear DNA.

Mahonia and *Berberis* are sister genera that share many similar morphological characteristics and often confuse together. So far, based on morphology, 2 genera were classified in 1 genus namely Berberis. So, in this study, ITS2-300bp could not clearly divide the species of these two genera into two separate groups is not surprising. This is also demonstrated that *Mahonia* and *Berberis* are closely genera with very small distance genetic.

From gotten result as above, we can see *rbc*L is a best DNA region can be used for identify species of Berberidaceae compare with *ITS*2 and *trn*H-*psb*A.

The *rbc*L DNA with 600 bp in length then was used to build phylogenetic trees for the 12 study samples, compare with other Berberidaceae species (data from GB) (Fig. 3). The results showed that 4 samples

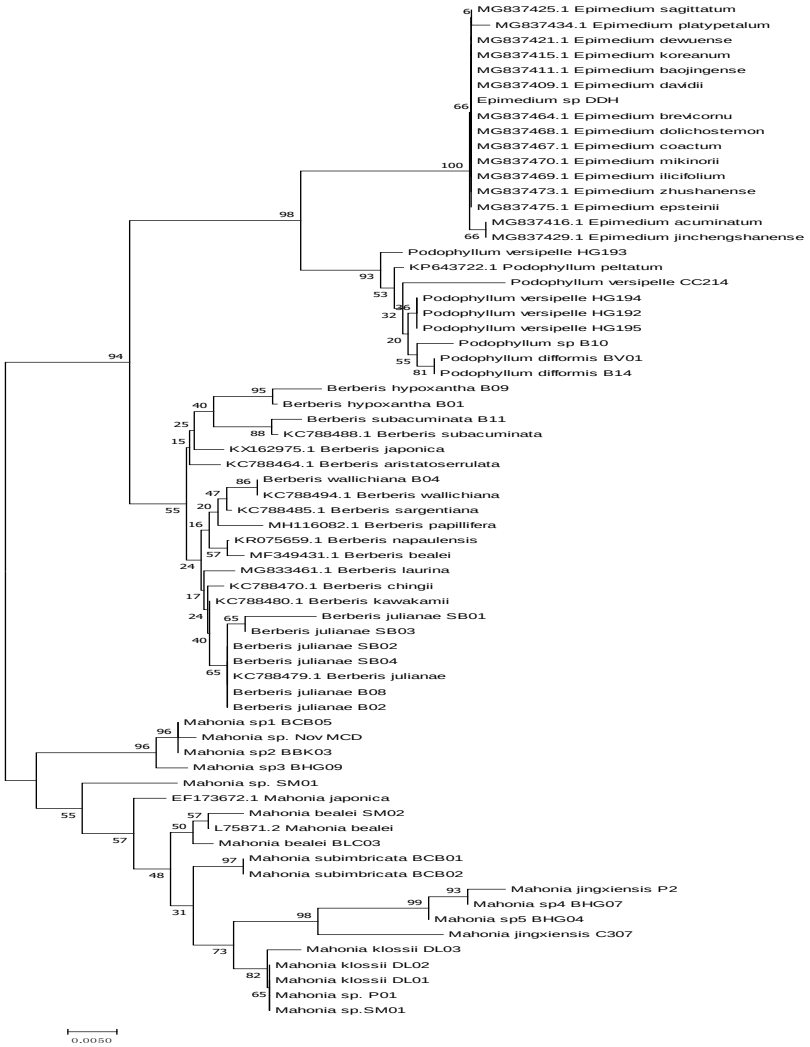


Fig. 3 Diagram of genetic relationship of the 12 studied species, compared with other species of Berberidaceae on the Gene Bank.

includes SB 01, SB 02, SB 03, SB 04 initially identified as species *Berberis julianae* gave 100% match with *Berberis julianae* species with the accession number KC788479.1, which proves that the initial morphological identification for these 4 species was completely accurate. The two samples BHG 07 and BHG 04 have not been accurately identified by morphology. After comparison, it is in the same clade with *Mahonia jingxiensis*, however the rate of similarity was only 98%. Following 3 species of BHG 09, BCB 05 and BBK 03 are also 3 species of *Mahonia* sp. but due to the lack of data on GB, so can not conclude exactly the scientific names for these 3 species, but based on genetic distance analysis, it has been shown that these 3 species have a close genetic relationship, they were classified in the same group in a branch of the genus *Mahonia* (Fig. 3).

4. Conclusion

All species of Berberidaceae have medicinal value and high economic value, they are exhausted in the wild. Currently, all of them are listed in the Red Book and Group IA of CITES (Conversion on International Trade in Endangered Species of Wild Fauna and Flora). In Berberidaceae, special in *Mahonia* and *Berberis* genera, they share many similarity of morphological characteristics (especially when plants are still in the immature stage), so it is really difficult to identify species based on morphology, so apply molecular biology techniques to identify species is necessary. The selection of the most suitable DNA barcoding markers for the classification of species of Berberidaceae has been done by us and the results showed that the *rbc*L region is suitable for studies on classifies and identifies Berberidaceaeat species level. Our study also showed that there is still lack of data on the DNA sequence of Berberidaceae species, so updating and adding molecular data about these species to have enough database for comparison, identification is also necessary and importance.

The sequence of ITS2 of *Berberis julianeae* and *Mahonia bealei* of Vietnam have been submitted to Genbank with accession number MT073031 and MT008067, respectively. The sequence of rbcL of Mahonia bealei also submitted to Genbank with accession number MT457415.1

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