TO CREATE DNA BARCODE DATA OF *MAGNOLIA CHEVALIERI* (DANDY) V.S. KUMAR FOR IDENTIFICATION SPECIES AND RESEARCHING GENETIC DIVERSITY

Ha Van Huan[1], Luu Thi Thao Nguyen[2], Nguyen Minh Quang[3]
[1,2,3]*Vietnam National University of Forestry*

SUMMARY

To create the nucleotide sequence of DNA barcode database, they are usually used as a DNA barcode to identify species and research of genetic diversity in plants. In this study, the genomic DNA was extracted from leaf tissue of *Magnolia chevalieri* (Dandy) V.S. Kumar. The DNA barcodes (*mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1b) were amplified from total DNA of *Magnolia chevalieri* (Dandy) V.S. Kumar by PCR technique. *DNA sequencing by Sanger method and* performed on an automated DNA sequencer. The DNA sequences were analyzed using software programs such as Mega6, BioEdit, GeneDoc, DNAClub and ClustalX. The PCR results indicated that all DNA bands were amplified from the samples, they have the same size similar to the theoretical size of *mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1b. Nucleotide sequence analysis of PCR product samples showed that the size of the isolated *mat*K gene fragment is 768 bp, *rbc*L *fragment is* 599 bp, *trn*H-*psb*A *fragment* is 512 bp, *ITS*2 fragment is 406 bp and *ycf1b* fragment is 1005 bp. The above nucleotide sequences were compared with the nucleotide sequences in the international gene bank NCBI, the highest level of similarity as follows: the *mat*K fragment and *rbc*L fragments compared with *Magnolia conifera var. chingii* species with 99.73% similarity and 99.63% similarity, respectively; the *trn*H-*psb*A compared with *Magnolia conifera var. chingii* pecies with 99.22% similarity and the*y cf*1b fragment compared with *Magnolia officinalis* subsp. Biloba species 99.5% similarity. The nucleotide sequences of *mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1b from *Magnolia chevalieri* (Dandy) V.S. Kumar have been registered on the DNABank.vn with Barcode ID MMC0001, MMC0002, MMC0003, MMC0004 and MMC0005. Recommendation for using *ITS*2 *andtrn*H-*psb*A marker as DNA barcode to identify *Magnolia chevalieri* (Dandy) V.S. Kumar species.

Keywords: DNA barcode, ITS2, *Magnolia chevalieri*, matK, rbcL, trnH-psbA, ycf1b.

I. INTRODUCTION

Magnolia chevalieri (Dandy) V.S. Kumar is belonging to *Magnoliaceae* which is considered as a high economic value wood trees in Vietnam. This type of wood has good quality so it is often used in home furniture, interior decoration and handicraft production (Wang et al., 2004). However, this species is on the list of plants that need to be conserved in the country. In Vietnam, *Magnolia chevalieri* distributes mainly in Phu Tho, Lao Cai, Vinh Phuc, Ha Noi, Quang Ninh, Tuyen Quang và Thanh Hoa provinces. *Magnolia chevalieri* is hardly found in natural forest, therefore, the conservation of *Magnolia chevalieri* genetic resources is essential.

Previously, identification and classification of plants were mainly based on morphological methods. Although there have been improvements in the application process, there are still many difficulties as in cases that the samples have been distorted (nature, colour changes), Processed samples can not be identified. In fact, the identification of some species belonging to Magnoliaceae based on morphological indicator sometimes faces with difficulties and can be confused. Hence, More accurate identification and classification methods are required to overcome these limitations (Chase et al., 2005; Group, 2009).

Recently, the development of DNA barcode technology application which is an advanced method, using the sequence of short DNA strands featured in the genome of the organism to identify and distinguish species, brings high efficiency in a short time, contributes to improve the drawbacks of the previous method (Ha Van Huan, 2015). In this study, we

conducted a selection of five specific DNA sequences to use as DNA barcodes which are: *mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf1*b. In which, *mat*K, *rbc*L, *trn*H-*psb*A, *ycf1*b sequencesare located in the chloroplast genome, *ITS*2 sequencein the nuclear gennome (Chase et al., 2005; Group, 2009; Kress et al., 2008; Kress et al., 2005; Spooner et al., 2009; Von et al., 2011). Hence, the study of DNA barcode data construction is used as indicative standard DNA molecule in order to serve the identification and study of genetic relationship of *Magnolia chevalieri* (Dandy) V.S. Kumaris necessary, contributes to natural resources management, national precious gene sources conservation and development.

II. RESEARCH METHODOLOGY

Objects, materials

Research Object: *Magnolia chevalieri* (Dandy) V.S. Kumar.

Research Materials: 3 young leaf samples were taken from 3 different trees of *Magnolia chevalieri* (Dandy) V.S. Kumar which were accurately identified by the scientific names. Once collected, samples were stored in plastic bags containing silica gel desiccant, then stored at - 20°C to extract DNA for research. Symbols of *Magnolia chevalieri* (Dandy) V.S. Kumar samples were taken in accordance with their abbreviations and scientific names of species: MMC1, MMC2, MMC3.

The primers were designed for cloning fragments of DNA sequences as in table 1.

Table 1. List of primers to clone the fragments of DNA barcode

Forward/Reverse Primers	Primer sequence (dimensional 5' - 3')	Cloned fragments of DNA barcode
*mat*K - mLKTF	5'- TTCCATGGCCTTCTTTBCATTTGTTGC - 3'	*mat*K
*mat*K - mLKTR	5'- TTCCATGGTTTTTTGAGGATCCGCTGT - 3'	
rP1F	5'- ATGTCACCACAAACAGAGACTAAAGC -3'	*rbc*L
rP1R	5'- GTAAAATCAAGTCCACCRCG -3'	
trnPF1	5'- GTTATGCATGAACGTAATGCTC -3'	*trn*H - *psb*A
psbPR1	5'- CGCGCATGGTGGATTCACAATCC -3'	

***Chemicals*:** The chemicals used to isolate the total DNA from *Calocedrus macrolepis* leaf samples: Plant DNA Isolation Kit of Norgen, Canada; Chemicals for PCR cloning fragments of DNA barcode: Master mix of *iNtRON* Biotechnology, Korea; PCR Purification Kit of Norgen, Canada; Chemicals for electrophoresis on Agarose gel: Agarose, 1 kb DNA Ladder, RedSafeTM nucleic acid staining solution provide by Norgen, Canada.

Research methods

Total DNA isolation

Total DNA is isolated from leaf samples of *Magnolia chevalieri* under the guidance of Plant DNA Isolation Kit, Norgen, Canada. Concentration, purity and integrity levels of total DNA are determined by spectrophotometric and electrophoresis methods on 1% agarose gel.

Cloning fragments of DNA barcodes by PCR technique

The fragments of DNA such as: *mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1 bare cloned by PCR technique on PCR 9700 Thermal Cycler Applied Biosystems (USA), each PCR reaction was performed in a total volume of 20 µl, including: H_2O deion (7 µl), 2x PCR Master mix Solution (10 µl), 10 pmol/µl of forward primers (1.0 µl), 10 pmol/µl of reverse primers (1.0 µl) and 50 ng/µl of DNA template (1 µl). PCR reaction program: 94°C in 2 minutes; (94°C: 30 seconds, 59°C: 30 seconds, 72°C: 1 minute) repeating 40 cycles; 72°C in 5 minutes; PCR product preservation is at 4°C. Primer melting temperature (Tm) of reactions are different depending on the used primers.

Each PCR reaction was repeated 3 times on each sample. PCR results aretested by electrophoresis on 1.2% agarose gel and observed under ultraviolet light (UV). PCR products are purified according to instructions of PCR Purification Kit of Norgen, Canada.

Identification and analysis of nucleotide sequences of cloned DNA fragments

PCR products cloned fragments of DNA barcodes after purification are sent to the 1 st Base lab in Malaysia for sequencing. The nucleotide sequence of the DNA fragment is determined by sequencer, using Kit BigDye® Terminator v3.1 Cycle Sequencing. The nucleotide sequences of DNA fragment are processed and analyzed using specialized software such as DNAClub, Biohit, Mega6... The nucleotide sequence of DNA barcode fragments after processing are registered in the Vietnamese DNA database bank (www.dnabank.vn).

III. RESULTS AND DISCUSSION

Result of total DNA extraction from *Magnolia chevalieri* (Dandy) V.S. Kumar leaves

Total DNA extracted from leaves of *Magnolia chevalieri* (Dandy) was tested by Electrophoresis on 1.0% agarose gel to preliminarily evaluate the content and quality. The result of electrophores is test shows that the DNA band is relatively sharp, with little break age, it proves that the total DNA is quite intact. The concentration and purity of the DNA solution is determined by Spectrophotometric method at A_{260nm} and A_{280nm} wavelengths. The result shows that the total DNA solution extracted from leaves of *Magnolia chevalieri* (Dandy) ensures the technical requirements to make molds for cloning the being interested DNA fragments.

Result of cloning DNA barcode fragments using PCR

Total DNA products after extracting and diluting to the appropriate concentration will be used directly as a mold for cloning *mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1b fragments using specific pairs of primers. The composition and reaction cycle are described in the method section. Each PCR reaction was repeated 3 times on each sample. PCR products were examined by electrophoresis on 1.2% agarose gel, using marker 1 kb. Electrophoresis The result shows that the corresponding DNA bands appear to the right size as expected:

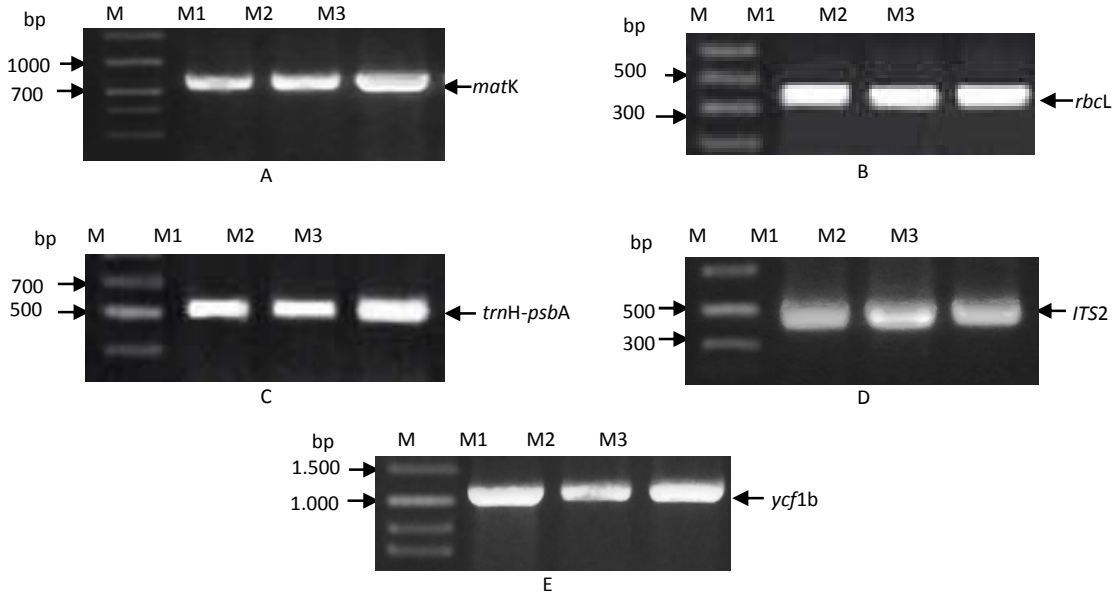

Figure 1. PCR result of DNA barcode fragments cloning

A. PCR result of *mat*K gene fragment cloning; B. *rbc*L; C. *trn*H - *psb*A; D. ITS2; E. *ycf1*b; M. DNA marker 1 kb of Norgen

Electrophores is result of PCR product off DNA barcode fragments on 1% agarose gel shows that DNA bands are bold, sharp, with no by products and right with the expected size, it proves that PCR products are specific, can be purified and directly used to proceed to determine nucleotide sequence of barcode fragments for *Magnolia chevalieri* (Dandy) V.S. Kumar.

The result of identifying and analyzing the nucleotide sequence of the DNA barcode

PCR products of DNA barcode fragments after purification were proceeded to determine the nucleotide sequence. The result of sequencing analysis shows that *mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1b fragments have the lengths of 768 bp, 599 bp, 512 bp, 406 bp and 1005 bp accordingly. The above result shows that the lengths of the corresponding DNA barcode fragments are equal to the expected length and band size on electrophores is gel. Comparing DNA sequences in all three iterations, we found no significantly difference, *mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1b sequence fragments were registed in Vietnamese DNA Data Bank (DNABank.vn) with the corresponding Barcode IDs: MMC0001, MMC0002, MMC0003, MMC0004 and MMC0005.

Table 2. The nucleotide sequence of the *mat*K gene fragment, including 768 nucleotides

Barcode ID: MMC0001

5’TTCCATGGCCTTCTTTGCATTTATTGCGATTCTTTCTCCACGAGTATCGTAATTCGAATAGTCT
CATTACTCCAAAGAAATCCATTTCTCTTTTTTCAAAAGAGAATCAAAGATTCTTCTTGTTACTA
TATAATTCTCATGTATATGAATGTGAATCCGTATTAGTGTTTCTCCGTAAACAATCTTCTCATTT
ACGATCAACATCCTCTGGAACTTTTCTTGAGCGAACACATTTCTATGGAAAAATAGAACATCT
TGTAGTAGTGCTTCGTAATGATTTTCAGAAGACCCTATGGTTGTTCAAGGACCCTTTCATGCAT
TATGTCAGATATCAAGGAAAATCCATTCTGGCTTCAAAGGGGACTCATCTTCTGATGAAGAAA
TGGAAATCTCACCTTGTCCATTTTTGGCAATGTCATTTTTACTTGTGGTCTCTACCGGACAGGA
TCCATATAAACCAATTATACAATCATTCCTTATATTTTCTGGGCTATCTTTCAAGTGTACGACT
AAACACTTCGGTGGTAAGGATTCAAATGCTAGAGAATTCATTTCTAATAGATACTTCTATTAAT
AAATTCGAGACCCTAGTCCCAATTATTCCTCTGATTGGATCAGTGGCTAAAGCGAAATTTTGTA
ACGTATCAGGGCATCCCATTAGTAAGTCGGTCCGGGCCGATTCGTCAGATTCTGATATTATCA
ATCGATTTGGGCGGATATACAGAAATCTTTCTCATTATCACAGTGGATCCTCAAAAAACCATG
GAA-3’

Table 3. The nucleotide sequence of the *rbc*L gene fragment, including 599 nucleotides

Barcode ID: MMC0002

5’ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGAGTACA
AATTGACTTATTATACTCCTGAATATGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAG
TAACTCCTCAACCCGGAGTTCCACCTGAGGAAGCAGGGGCTGCGGTAGCTGCCGAATCTTCTA
CTGGTACATGGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGAT
GCTACCACATCGAGCCCGTTCCTGGGGAGGAAAGTCAATTTATTGCTTATGTAGCTTACCCTTT
AGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTAGGTAATGTATTTGGG
TTCAAAGCCCTACGAGCTCTACGTCTGGAGGATCTGCGAATTCCTACTGCTTATGTCAAAACTT
TCCAAGGCCCGCCCCATGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCAC
TATTGGGATGTACTATTAAACCAAAATTGGGGTTATCCGCCAAGAACTACGGTAGGGCGGTTT
ATGAATGTCTCCGTGGTGGACTTGATTTTAC-3’

Table 4. The nucleotide sequence of the *trn*H-*psb*A gene fragment, including 512 nucleotide

Barcode ID: MMC0003

5’CGCGCATGGTGGATTCACAATCCACTGCCTTGAGCCACTTGGCTACATCCGCCCCTCCGCTCT
AATTTCCACCATTCATGATTATTGTATTGAGTCTTTCTTACTTTCTGAGATACAGATATTGAAC
ATAAAATGCCAATCCTGTAAATTGTAAATGTACAAAGAAATTCCTTTATGAAAAAAAAAAAG
AAAATGATTTTGAGGAACATACAGAAATTACAATACAGATCGGTACAAAACAAAAATAGGAT
GTTCGATCATGAACCAACCAACAATAATGTTTTCTTAAGTTGAAATAAAGAAATGAAAATGGC
AAAAATGTTTCTGTGAATAAAACACTACTGAATCGAACGGATCAATACCCAACTTCTTGATAG
AACAAGAAGTTGGGTATTGATCGGATCCTTCAACGACTCATATACACTAAGACTGAAGTATTA
TCCATTTGTAGATGGAACTTCAACAGCAGCTAGGTCTAGAGGGAAATTGTGAGCATTACGTTC
ATGCATAAC-3’

Table 5. The nucleotide sequence of the ITS2 gene fragment, including 406 nucleotide

Barcode ID: MMC0004

5’ATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGC
GCCCGAGGCCACCCGGCCGAGGGCACGCCTGCCTGGGCGTCACGCACCGTGCTGCCCCACCCG
GCGCCCGCCCCACCCGGCGGCGGCGCCGCGGGGCGGAGACTGGCCGCCCGTGCGCCCCGCGC
GCGCGGCCGGCTGAAAAGCATTCGCTCCCCCCCGCCGGGGCGCGGACGCGGCGGTAGGTGGT
TTGAGAGGCGGCTGCCTCGTCGGAGCCCGGACGCCGCGCCCGTCGCCCCGCGCGGCGAAGTG
GCACCCAGCTCGGCCGCCCCCGCGCGGCCCTCGCGCAGCGACCCCAGGTCAGGCGGGGACAC
CCGCTGAGTTTAAGCATATCAATAAGCGGAGGA-3’

Table 6. The nucleotide sequence of the *ycf*1b gene fragment, including 1005 nucleotide

Barcode ID: MMC0005

5’ACGAAAATCCGATTGTTGCGAGTAACGTATCAACGCCACCTCTTCTGCTTGATCACTATTAG
TACTGGTACTAGTATAAGTATTGGTATTCTGATCATTATTGGTATTCTGATCATTATTGGTATTC
TGATCATTATTGGTATTCTGATCATTATTGGTATTCTGATCATTATCAGTATAAATTACTACAC
GTTTGGCTTTTCTTGAACGAATCCCATGATCCTCTGTCGATTCTTCCTCATTTTCTGCCTCCTGT
TCTTCCAAATGGCCGGTTAATTTGTATGACCATCGAGGAACCTTTTTACCGATTTCTTCTATTCC
AATAGATTTCTTTTGAATTGTTTGATCATTTGGATCAGTTGTAACTACATCGGATAGAAATTTC
AAACATTTCGTTTGATTTTCTGAATCAAGTCTTGTTTGTTCGACCAATAGAGCAAATCCTTTCA
AATTCAAACTAGGTGTTGATTCCCCAGCTAATTCACTGATTGAGGTCAAGGAATGACCAATGT
CTGTCGATAATGATTCTCCATTAAATAAATCCATTTTTTGTTCAAATTCTCGGTAATCGTTAGG
AAAGATACCATGAATCTTATTTATCCAAACCTTTTCTATGGAATCTTCTGTCCAAGTGATTGAA
TCATCCATAATTGAACGTGAATATCCTTTTTTGGTTGTTCCACGATATGATCCGTTCAAAAAAG
GATCATATATTTTAGGCAAGCATTCTTGTTCATTCTCATTATTGCACAATCTTGTCCTTTTTTCG
AGCACATCCAGAGTAAGAGTTCCCCTGTCTAGGGCTTCTATTCGATTTACGAACTCATTGCTCA
AGTTGTTCCTTTTTTGTTCATTGGTAGAAACCCAATGATTATACAGATCCTCCGGGGATAATAG
TTTTTCTGTCGTACGCAAAGACATCTTTCTTTGTATCATTTCCCCAAAAGTCGACAAACTAGGG
GGATATGTAAAAGATATTCGTTTTTTTCCATCACTTGGACATGT-3’

We conducted a comparison of the nucleotide sequences of DNA barcode fragments (*mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1b) of *Magnolia chevalieri* (Dandy) V.S. Kumar with the nucleotide sequence of the corresponding barcode fragments of other *Magnolia* sp. on the NCBI international gene bank, then selected the species with the highest similarity in nucleotide sequence, the results are shown in table 7.

Table 7. Comparing the nucleotide sequence of the barcodes *mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1b of *Magnolia chevalieri* Dandy V.S. Kumar with the nucleotide sequence of the corresponding barcode fragments of other *Magnolia* sp. published on the NCBI

No	DNA barcode fragment	Species for comparison	NCBI Accession No.	Differential nucleotides	Simil-arity (%)
1	*mat*K	*Magnolia conifera* var. chingii	JN050058.1	2	99.73
2	*rbc*L	*Magnolia conifera* var. chingii	JN50121.1	2	99.63
3	*trn*H-*psb*A	*Magnolia conifera* var. chingii	JN05177.1	3	99.22
4	*ITS*2	*Magnolia figo var. skinneriana*	KP092910.1	11	96.97
5	*ycf*1b	*Magnolia officinalis* subsp. *biloba*	JN867581.1	5	99.5

Results of the analysis showed that when comparing the sequence of 5 fragments of DNA *mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1b of *Magnolia chevalieri* (Dandy) V.S. Kumar with other species in the same genus, all DNA fragments have a definite difference compared to those published in the NCBI gene bank, hence, all 5 of these fragments are valid for use. However, the degree of variation of the indicators is different, the larger indicator's difference, the greater its value, in which *ITS*2 indicator has the most difference (3.03%), followed by *trn*H-*psb*A, *ycf1*b, *rbc*L and *mat*K. From the result of the analysis of DNA barcode nucleotide sequence, we recommend using *ITS*2 and *trn*H-*psb*A indicators to identify anddetermine the genetic relationship for *Magnolia chevalieri* (Dandy) V.S. Kumar, however, to increase accuracy, they can be combined with other indicators such as *ycf1*b, *rbc*L and *mat*K.

IV. CONCLUSION

Successfully cloned *mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1b sequence fragmentsby PCR technique. Sequence analysis result shows that the lengths of *mat*K, *rbc*L, *trn*H-*psb*A, *ITS*2 and *ycf*1b fragments are 768 bp, 599 bp, 512 bp, 406 bp and 1005 bp accordingly. The nucleotide sequences of the above barcodes have been registered on the DNA database of Vietnam with corresponding barcode IDMMC0001, MMC0002, MMC0003, MMC0004 and MMC0005. The result of the sequential comparison of the DNA fragments shows that the *mat*K fragment has a similarity of 99.73%, followed by *rbc*L, *trn*H-*psb*A 99.63%, 99.22% respectively compared to *Magnolia conifera* var. chingii; *ITS*2 fragment has a similarity of 96.97% with *Magnolia figo* var. skinneriana and *ycf*1b fragment has 99.5% similarity with *Magnolia officinalis* subsp. biloba. Using *ITS*2 and *trn*H-*psb*A indicators to identify and determine the genetic relationship for *Magnolia chevalieri* (Dandy) V.S. Kumar, however, to increase accuracy, they can be combined with other indicators such as *ycf1*b, *rbc*L and *mat*K.

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XÂY DỰNG DỮ LIỆU DNA BARCODE CHO LOÀI MỠ PHÚ THỌ (*MAGNOLIA CHEVALIERI* (DANDY) V.S. KUMAR) PHỤC VỤ GIÁM ĐỊNH VÀ NGHIÊN CỨU ĐA DẠNG DI TRUYỀN

Hà Văn Huân[1], Lưu Thị Thảo Nguyên[2], Nguyễn Minh Quang[3]

[1,2,3]Trường Đại học Lâm nghiệp

TÓM TẮT

Nhằm tạo dữ liệu DNA là trình tự nucleotide của một số đoạn ADN đặc trưng thường được sử dụng làm mã vạch để phục vụ giám định và nghiên cứu đa dạng di truyền ở thực vật. Nghiên cứu này đã tiến hành phân lập và xác định trình tự nucleotide của năm đoạn ADN là: *mat*K, *rbc*L, *trn*H-*psb*A, ITS2 và *ycf*1b ở loài Mỡ phú thọ. Kết quả xác định trình tự nucleotide của các đoạn mã vạch ADN, như sau: đoạn *mat*K có chiều dài 768 nucleotide, đoạn *rbc*L là 599 nucleotide, đoạn *trn*H-*psb*A là 512 nucleotide, đoạn ITS2 là 406 nucleotide và đoạn *ycf*1b là 1005 nucleotide. So sánh trình tự nucleotide của các đoạn trên với các trình tự nucleotide tương ứng trên ngân hàng gen quốc tế NCBI, chọn trình tự của loài có độ tương đồng cao nhất như sau: đoạn gen *mat*K có độ tương đồng 99,73%, đoạn *rbc*L tương đồng 99,63%, đoạn *trn*H-*psb*A tương đồng 99,22% so với loài *Magnolia conifera var. chingii;* đoạn ITS2 tương đồng 96,97% so với loài *Magnolia figo* var. skinneriana và đoạn *ycf*1b tương đồng 99,5% so với loài *Magnolia officinalis* subsp. biloba. Trình tự nucleotide các đoạn mã vạch trên đã được đăng ký trên ngân hàng dữ liệu ADN Việt Nam với các mã số (Barcode ID) tương ứng là MMC0001, MMC0002, MMC0003, MMC0004 và MMC0005. Như vậy, cả 5 đoạn mã vạch ADN trên đều có sự khác biệt nhất định so với các loài đã công bố trên Ngân hàng gen quốc tế NCBI trong đó đoạn ITS2 có khả năng phân biệt tốt nhất.

Từ khóa: ITS2, mã vạch ADN, matK, Mỡ phú thọ, rbcL, trnH-psbA, ycf1b.

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