Identification of dna barcode sequence of hybrid eucalyptus UP99 (*E. urophylla* x *E. pellita*) and UP95 (*E. urophylla* x *E. pellita)* to identify plant varieties

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Xác định DNA mã vạch giống bạch đàn lai UP99 (*E. urophylla* x *E. pellita*) và UP95 (*E. urophylla* x *E. pellita*) phục vụ giám định giống cây

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ABSTRACT

The hybrid Eucalyptus UP99 (E. urophylla x E. pellita) and UP95 (E. urophylla x E. pellita) were recognized as a high economic value species according to Decision 65/QĐ-BNN-LN on January 11th in 2013. However, it is very difficult to the famer in identifying these species by morphological observation. Therefore, this study aimed to develop a method using DNA barcode fragments to identify the hybrid Eucalyptus UP99 and UP95. The total genomic DNA was extracted from leaf samples of UP99 and UP95 and was used to amplify the DNA barcodes (matK, rbcL, trnH-psbA, ITS and ITS2) by PCR. The results showed that the bands of PCR production have the expected size, which is 643 bp, 743 bp, 626 bp, 563 bp, and 250 bp for matK, rbcL, trnH-psbA, ITS, and ITS2 fragment, respectively. After that, these sequences were aligned with the sequence of those genes of other Eucalyptus species in NCBI. The results showed that UP99 with matK, rbcL and trnH-psbA gene fragments are 100% similar to UP95, ITS gene fragment is 99.81% similar to UP95, ITS2 gene fragment is 98.86% similar to UP95. These results suggest that it is best for using ITS2 and ITS molecular marker as a DNA barcode to identify Hybrid Eucalyptus UP99 (E. urophylla x E. pellita) and UP95 (E. urophylla x E. pellita) in Vietnam. These results are an important basis for the identification hybrid Eucalyptus UP99 (E. urophylla x E. pellita) and UP95 (E. urophylla x E. pellita).

Từ khóa:
Bạch đàn lai UP99, Bạch đàn lai UP95, DNA mã vạch, giám định loài, PCR.

TÓM TẮT

Giống Bạch đàn lai UP99 (E. urophylla x E. pellita)và UP95 (E. urophylla x E. pellita) được công nhận là giống có giá trị kinh tế cao theo quyết định 65/QĐ-BNN-LN ngày 11/1/2013. Tuy nhiên, đối với những người nông dân việc xác định giống chỉ bằng quan sát hình thái là hết sức khó khăn. Do đó, mục đích của nghiên cứu này là sử dụng DNA mã vạch để xác định giống Bạch đàn lai UP99 và UP95. DNA tổng số được tách chiết từ các mẫu lá của UP99 và UP95 và được sử dụng để nhân bản các đoạn gen matK, rbcL, trnH-psbA, ITS và ITS2 bằng kỹ thuật PCR. Các kết quả chỉ ra rằng các băng của sản phẩm PCR đúng với kích thước dự kiến như 643 bp, 743 bp, 626 bp, 563 bp và 250 bp, tương ứng với các đoạn gen matK, rbcL, trnH-psbA, ITS, và ITS2. Sau đó, các trình tự này được so sánh với các trình tự trên ngân hàng gen quốc tế. Kết quả đã chỉ ra rằng giống Bạch đàn lai UP99 và UP95 có tỷ lệ tương đồng 100% ở đoạn gen matK, rbcL và trnH-psbA, tương đồng 99,81% ở đoạn gen ITS, tương đồng 99,86% ở đoạn gen ITS2. Kết quả cũng cho thấy sử dụng chỉ thị ITS và ITS2 làm DNA mã vạch để giám định giống Bạch đàn lai UP99 và UP95 là tốt nhất. Kết quả nghiên cứu là cơ sở quan trọng cho việc xác định giống Bạch đàn lai UP99 và UP95 đang trồng ở nước ta.

1. INTRODUCTION

Eucalyptus belongs to Myrtaceae which has a large number of species. They are timber trees of high economic value species. The wood of these species has good quality to be used in house furniture, raw materials for pulp, plywood in industry and eucalyptus oil to treat headaches, bone pain [1, 2]. In this study, the two varieties of hybrid Eucalyptus were chosen as shown in table 1. All of them are fast growth, good resistance and high productivity which has high economic value in planting forests. However, it is very hard for famer in identifying these species by morphological observation. Previously, identification and classification of plants vere mainly based on morphological methods. Therefore, nowadays, DNA barcoding is molecular method which helps to identify the organisms based on short, standardized gene sequences in nuclear genome, chloroplast genome, mitochondrial genome of organisms in the short time and accurate efficiency [7]. DNA barcoding is effective tool which improves the drawbacks of the morphological methods [5]. DNA barcodes were used for the classification and identification of all organisms, including plants, animals, fungus, microorganisms and viruses. The short gene sequences in DNA barcodes were located in the nuclear genome (*ITS*, 5.8S, 18S…), in which these sequences show significant sequence variability at the species level or subspecies. The short gene sequences in DNA barcodes were also located in the chloroplast genome (*mat*K, *rbc*L, *trn*H-*psb*A, *ycf*1b…) and mitochondral genome (*Cyt*b, *CO*1...), in which these sequences have high conservation, suitable to DNA barcode in plants [6, 9-12].

In this study, we selected the five candidate DNA barcode regions, including 3 regions (*mat*k, *rbc*L, *trn*H-*psb*A) are located in the chloroplast genome and two regions (*ITS*, *ITS*2) are located in nuclear genome. Using these fragment sequences can be bring positive results to classification, identification as well as the study of genetic relationship of plants, contributing to improve efficiency conservation and development.

2. RESEARCH METHODOLOGY

2.1. Plant materials

The leaves of UP99 and UP95 were collected at Experimentation center and transfer of Forestry Variety, Bavi district. Each species got three samples of different individual plants. These samples were kept in silica gel and stored at -80°C. The samples of UP99 and UP95 were labled as show the Table 1.

Table 1. The information of variety

Order	Scientific names	Symbol of varieties
1	*E. urophylla* x *E. pellita* (UP99)	UP99.1; UP99.2; UP99.3
2	*E. urophylla* x *E. pellita* (UP95)	UP95.1; UP95.2; UP95.3

2.2. Chemical materials

Plant DNA isolation Kit of Norgen, Canada; Master mix of intron biotechnology, Korea; PCR purification Kit of Norgen, Canada; Agarose; 1Kb DNA ladder; Redsafe of Norgen, Canada. The primers were designed for amplification of DNA barcode sequences as in the Table 2 [4].

Table 2. The list of primers

DNA barcode locus	Primers	Primer sequence (5'-3')	Temperature (°C)
*mat*K	mP3F	TTCCATGGCCTTCTTTGCATTTGTTGC	50°C
	mP3R	TTCCATGGTTTTTTGAGGATCCGCTGT	
*rbc*L	rP2F	TGTCACCACAAACAGAGACTAAAGC	52°C
	rP2R	GTAAAATCAAGTCCACCTCG	
*trn*H-*psb*A	*trn*PF1	CGCGCATGGTGGATTCACAATCC	51°C
	*psb*PR1	GTTATGCATGACGTAATGCTC	
ITS	ISP2F	CGAATTCATGGTCCGGTGAAGTGTTCG	50°C
	ISP2R	AGAATTCCCCGGTTCGCTCGCCGTTAC	
*ITS*2	Is2P1F	ATGCGATACTTGGTGTGAAT	48°C
	Is2P1R	TCCTCCGCTTATTGATATGC	

2.3. Methods

Total DNA was extracted by plant DNA isolation kit, Norgen, Canada. The DNA barcode fragments (*mat*K, *rbc*L, *trn*H-*psb*A, *ITS* and *ITS*2) were amplified by PCR technique on PCR 9700 thermal cycler Applied Biosystems (USA). The PCR reaction included: deionized water (7µl); 2xPCR Master mix solution (10µl); 10pmol/µl Forward primer (1µl); 10pmol/µl Reverse primer (1µl) and 50ng/µl DNA template (1µl). The PCR reaction program: 94°C in 5 min; (94°C in 30sec; 48°C-52°C in 30sec; 72°C in 1 min) repeated 40 cycles; 72°C in 5 min; incubated at 4°C. Each PCR reaction was repeated 3 times for each sample. The PCR products were purified by PCR purification kit. After that, these products were sequenced by Sanger's method, using kit BigDye Terminator v3.1 Cycle Sequencing. The DNA sequences were analyzed by different softwares such as MegaX, Bioedit, NCBI.

3. RESULTS

3.1. Total DNA extraction

Total DNA were extracted from leaves of UP99 and UP95, and then total DNA products were tested by electrophoresis on 1% agarose gel. The results of electrophoresis showed that all DNA bands were clear and not breakage. Therefore, the total DNA products were suitable for using as DNA template for PCR reaction.

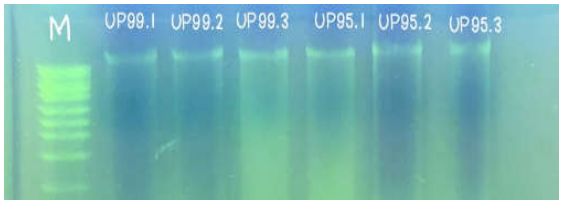

Figure 1. Agarose gel electrophoresis of the total crude DNA extracted from UP99 and UP95
UP99: UP99.1; UP99.2; UP99.3 (UP99 was repeated 3 times)
UP95: UP95.1; UP95.2; UP95.5 (UP95 was repeated 3 times)

3.2. PCR amplification

Total DNA of UP99 and UP95 were used to be templates in PCR reaction to amplify DNA fragments (*mat*K, *rbc*L, *trn*H-*psb*A, *ITS* and *ITS*2) with specific primers. The PCR results were tested by electrophoresis on agarose 1% (Fig. 2; Fig. 3).

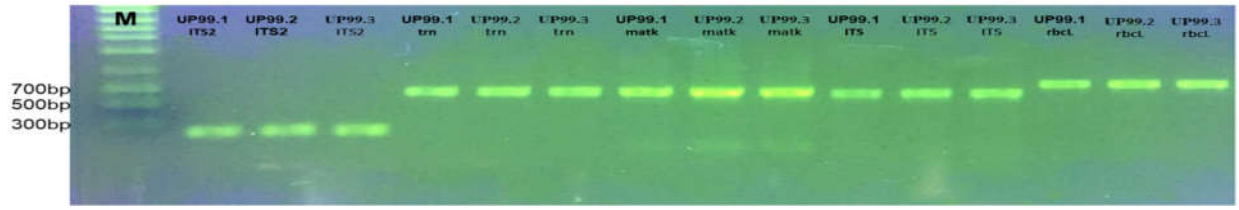

Figure 2. Agarose gel electrophoresis of PCR products from UP99 (UP99.1; UP99.2; UP99.3) with DNA barcodes (*ITS*2, *trn*H-*psb*A, *mat*K, *ITS* and *rbc*L)

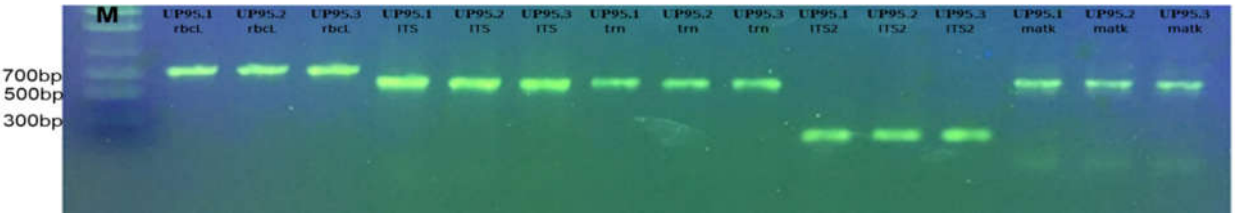

Figure 3. Agarose gel electrophoresis of PCR products from UP95 (UP95.1; UP95.2; UP95.3) with DNA barcodes (*ITS*2, *trn*H-*psb*A, *mat*K, *ITS* and *rbc*L)

The DNA bands were clear, no by-product, which showed that the primers were specificity. These results indicated that the size of five DNA barcodes: *ITS*2, *trn*H-*psb*A, *mat*K, *ITS* and *rbc*L were the expected size as 250bp, 626bp, 643bp, 563bp, 743bp, respectively. These PCR products were sequenced directly after which was purified by PCR purification kit (Norgen-Canada).

3.3. The DNA sequence analysis of five DNA barcodes

3.3.1. The DNA sequence of rbcL fragment

The DNA sequences of clones from the *rbc*L PCR fragments of UP99 and UP95 were 743bp and 687bp, respectively (Fig.4; Fig.5). There was no difference among three repetitions of UP99 and UP95.

ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGATTATAAACTGACTTATTATACTCCTGACTATG
AAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCCTCCTGAGGAAGCAGGGGCTGCGGTAGCTGCTG
AATCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGCCTTGATCGTTATAAAGGAAGATGCTACCACATCGAGCCTG
TTGCTGGAGAAGAAAATCAATATATATGTTATGTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAATATGTTTACTTCCATT
GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTGGAGGATCTGCGAATCCCTCCTTCCTATACGAAAACTTTCCAAGGCC
CGCCTCATGGCATCCAAGTTGAGAGAGATAAATTGAACAAATATGGGCGTCCCCTATTGGGATGTACTATTAAACCGAAATTGGGGTTAT
CCGCTAAGAACTACGGTAGAGCAGTTTATGAATGTCTTCGTGGTGGACTTGATTTTACGAAAGATGATGAGAACGTGAACTCACAACCAT
TTATGCGTTGGAGAGACCGTTTCTTATTTTGTGCCGAAGCCATTTTTAAATCACAGGCTGAAACAGGTGAAATCAAAGGGCATTACTTGAA
TGCTACTGCAGGTACATGCGA

Figure 4. The *rbc*L fragment sequence of UP99

GGTGTTAAAGATTATAAACTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACTCC
TCAACCTGGAGTTCCTCCTGAGGAAGCAGGGGCTGCGGTAGCTGCTGAATCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGG
CTTACCAGCCTTGATCGTTATAAAGGAAGATGCTACCACATCGAGCCTGTTGCTGGAGAAGAAAATCAATATATATGTTATGTAGCTTA
CCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAATATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCT
ACGTCTGGAGGATCTGCGAATCCCTCCTTCCTATACGAAAACTTTCCAAGGCCCGCCTCATGGCATCCAAGTTGAGAGAGATAAATTGA
ACAAATATGGGCGTCCCCTATTGGGATGTACTATTAAACCGAAATTGGGGTTATCCGCTAAGAACTACGGTAGAGCAGTTTATGAATG
TCTTCGTGGTGGACTTGATTTTACGAAAGATGATGAGAACGTGAACTCACAACCATTTATGCGTTGGAGAGACCGTTTCTTATTTTGTG
CCGAAGCCATTTTTAAATCACAGGCTGAAACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCA

Figure 5. The *rbc*L fragment sequence of UP95

These sequences were compared to other species on NCBI to find the differences at species level. Some species had the similar sequences with UP99 as in Table 3.

Table 3. Some species are homology to UP99 on the *rbc*L fragment

Order	Scientific name	Code	Similarity ratio (%)
1	*E. urophylla*	KJ440000.1	100
2	*E. grandis*	AB537496.1	100
3	*E. pellita*	KF496742.1	100
4	*UP95_ E. urophyla x E. pellita*	UP95	100
5	*Syzygium aromaticum*	NC_047249.1	99.07

Using Mega X software to construct the phylogenetic tree and genetic distance based on *rbc*L fragment of UP99 with other species in the Table 3 as showed Fig.6 and Table 4.

Figure 6. Phylogenetic tree were built based on *rbc*L fragment sequence

Table 4. The genetic distances of UP99 with other species on *rbc*L fragment sequence

Scientific name	*UP 99_ E.urophyla x E.pellita*	*UP95_ E.urophyla x E.pellita*	*Syzygium aromaticum*	*E. urophylla*	*E. pellita*	*E. grandis*
UP99_*E.urophya x E.pellita*						
UP95_ E.urophyla x E.pellita	0.0000					
Syzygium aromaticum	0.0093	0.0093				
E. urophylla	0.0000	0.0000	0.0093			
E. pellita	0.0000	0.0000	0.0093	0.0000		
E. grandis	0.0000	0.0000	0.0093	0.0000	0.0000	

The results of phylogenetic tree combined with genetic distances and similarity ratio on *rbc*L fragment sequence which showed that UP99, UP95, *E. urophylla, E. grandis and E. pellita* were no difference with the highest similarity ratio of 100% (genetic distances were 0,0000), but there was the difference among UP99 and *Syzygium aromaticum* with similarity ratio of 99.07% (genetic distances was 0,0093). Therefore, the *rbc*L fragment had not yet determined the difference between the UP99 and UP95.

3.2. The DNA sequence of *mat*K fragment

The DNA sequences of clones from the *mat*K PCR fragments of UP99 and UP95 were 643bp in the length (Fig.7; Fig.8). And there was no difference among three repetitions of UP99 and UP95.

TGGCTTCAAAAGATACGCCTCTTCTGATGAAGAAATGGAAATATTACCTTGTTAATTTATGGCAATATCATTTTTACGCCTGGTTTCAAC
CAGGAAGGATCGATATAAACCAATTATGCAAGTATTCTCTTTACTTTTTGGGCTATCGTTCAAGCGTGCGACTAAATTCTTCAGTGGTACGAA
GTCAAATGCTAGAAAATTCATTTCTAATAAATAATGCTATGAAGAAGTTCGAGACAATAGTTCCAATTATTCCTCTGATTGGATCATTGTCTA
AAGCGAATTTTTGTGACACATTAGGGCATCCCATTAGTAAACCGACCCGGGCTGATTCATCAGATTCTGATATTATCGACCGTTTTTTGCGTA
TATCCAGAAATCTTTCTCATTATCACAGCGGATCCTCAAAAAAAAAAGAGTTTATATCGAGTAAAATATATACTTCGACTTTCTTGTGTTAAAA
CTTTGGCTCGTAAACACAAAAAGACTGTACGTACTTTTTTAAAAAGATTAGGTTCGGAATTTTTGGAAGAATTCCTTACGGAGGAAGAAGTT
GTTCTTTCTTTGATCTTCCCAAGAACTTATTCTACTTCACGAAGGTTATATAGAGGGCGGATTTGGTATTTGGATATTACTTCTATCAA

Figure 7. The *mat*K fragment sequence of UP99

TGGCTTCAAAAGATACGCCTCTTCTGATGAAGAAATGGAAATATTACCTTGTTAATTTATGGCAATATCATTTTTACGCCTGGTT
TCAACCAGGAAGGATCGATATAAACCAATTATGCAAGTATTCTCTTTACTTTTTGGGCTATCGTTCAAGCGTGCGACTAAATTCTTCA
GTGGTACGAAGTCAAATGCTAGAAAATTCATTTCTAATAAATAATGCTATGAAGAAGTTCGAGACAATAGTTCCAATTATTCCTCTG
ATTGGATCATTGTCTAAAGCGAATTTTTGTGACACATTAGGGCATCCCATTAGTAAACCGACCCGGGCTGATTCATCAGATTCTGAT
ATTATCGACCGTTTTTTGCGTATATCCAGAAATCTTTCTCATTATCACAGCGGATCCTCAAAAAAAAAGAGTTTATATCGAGTAAAAT
ATATACTTCGACTTTCTTGTGTTAAAACTTTGGCTCGTAAACACAAAAAGACTGTACGTACTTTTTTAAAAAGATTAGGTTCGGAATT
TTTGGAAGAATTCCTTACGGAGGAAGAAGTTGTTCTTTCTTTGATCTTCCCAAGAACTTATTCTACTTCACGAAGGTTATATAGAGGG
CGGATTTGGTATTTGGATATTACTTCTATCAA

Figure 8. The *mat*K fragment sequence of UP95

And then these sequences were compared to other species on NCBI to find the differences among UP99 and other species as in the Table 5.

Table 5. Some species are homology to UP99 on the *mat*K fragment

Order	Scientific name	Code	Similarity ratio (%)
1	*E. urophylla*	KJ510901.1	100
2	*E. grandis*	MG925369.1	99.45
3	*E. pellita*	KT633046.1	99.45
4	*UP95_ E. urophyla x E. pellita*	UP95	100
5	*Syzygium paniculatum*	KM065365.1	98.07

Using mega X we constructed the phylogenetic tree (Fig.9) and genetic distance (Table 6) to find the genetic relationship of UP99 with other species.

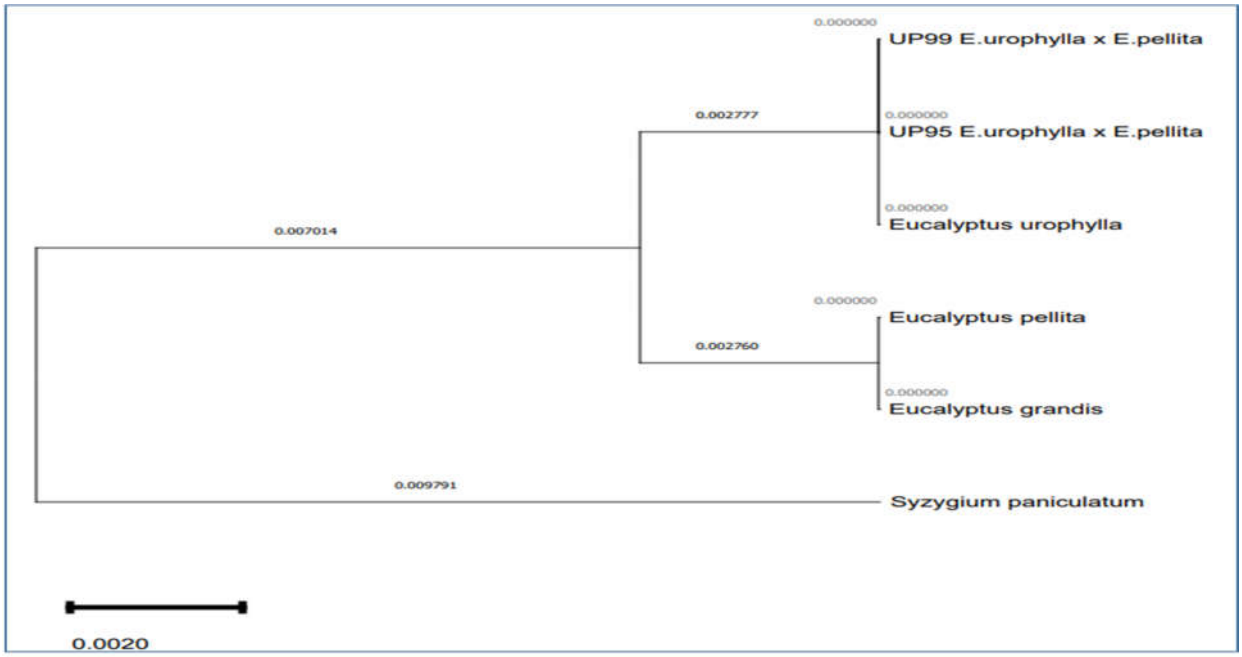

Figure 9. Phylogenetic tree were built based on the *mat*K fragment sequence

Table 6. The genetic distances of UP99 with other species on *mat*K fragment sequence

Scientific name	*UP99_E.urophylla x E.pellita*	*UP95_E.urophylla x E.pellita*	*Syzygium paniculatum*	*E. urophylla*	*E. pellita*	*E. grandis*
UP99_E.urophylla x E.pellita						
UP95_E.urophylla x E.pellita	0.0000					
Syzygium paniculatum	0.0196	0.0196				
E. urophylla	0.0000	0.0000	0.0196			
E. pellita	0.0055	0.0055	0.0196	0.0055		
E. grandis	0.0055	0.0055	0.0196	0.0055	0.0000	

Combining the phylogenetic tree with genetic distances and similar ratio based on *mat*K fragment showed that UP99, UP95 and *E. urophylla* had a the highest sequence similarity up to 100%. There was a little difference between UP99 to *E. grandis and E. pellita* with similarity ratio of 99,45% (genetic distances were 0.0055). However, UP99 had the highest difference with *Syzygium paniculatum* (genetic distance was 0.0196). So, the *mat*K fragment sequence had not yet determined the difference between two hybrid Eucalyptus UP99 and UP95.

3.3. The DNA sequence of *trn*H-*psb*A fragment

The *trn*H-*psb*A fragment sequence analysis of UP99 and UP95 were determined 626b p in the length (Fig.10; Fig.11). The three repetitions of UP99 and UP95 did not differ.

CGCGCATGGTGGATTCACAATCCACTGCCTTGATCCACTTGGCTACATCCGCCCCCACTACTACTAATATTCTTTTTTTTCTTTTTAAAT
GGATTAAAAAGAAAAAAAAGAATATTCCATTTTTAATGAAATAAAAAAAAGAAATTCATAATGGAAAATATTTCATTCGATTGTTAATTT
TTAACATTTTTCTATACTTAATTATGAGTAACATTTTTCTATCTTAATTATGAGATAGAAGAAGCAGAAAATTATAACCTTTCTATTTTATT
TGATAAAAAAAAACTAGAAGATAATAATCTCACAAAGCCTTACAAAGGGTTGAAAAGAATGTATATAAATTCATATCTAAGGAAAAAAG
TATGATAAGCAATCATAAAGCAATCCCTAAGACTAGAATACTTTTTCTTATGTTGAAGTAAAGAAAAACTTATGTAAAGAAAAGAGCACT
AAATAAAGGAACAATAACCAATTTCTTTTTCTATCAAGAGTGTTGGTTATTGCTCCTTTCCAATCAAAAACTCGGCTAGACTTATACTAAG
ACCAAAGTCTTATCCATTTGTAGATGGAACTTCGACAGCAGCTAGGTCTAGAGGGAAGTTATGAGCATTACGTTCATGCATAAC

Figure 10. The *trn*H-*psb*A fragment sequence of UP99

CGCGCATGGTGGATTCACAATCCACTGCCTTGATCCACTTGGCTACATCCGCCCCCACTACTACTAATATTCTTTTTTTTCTTTTT
AATCCATTTAAAAAGAAAAAAAAGAATATTCCATTTTTAATGAAATAAAAAAAAGAAATTCATAATGGAAAATATTTCATTCGATT
GTTAATTTTTAACATTTTTCTATACTTAATTATGAGTAACATTTTTCTATCTTAATTATGAGATAGAAGAAGCAGAAAATTATAACCT
TTCTATTTTATTTGATAAAAAAAAACTAGAAGATAATAATCTCACAAAGCCTTACAAAGGGTTGAAAAGAATGTATATAAATTCATA
TCTAAGGAAAAAAGTATGATAAGCAATCATAAAGCAATCCCTAAGACTAGAATACTTTTTCTTATGTTGAAGTAAAGAAAAACTTAT
GTAAAGAAAAGAGCACTAAATAAAGGAACAATAACCAATTTCTTTTTCTATCAAGAGTGTTGGTTATTGCTCCTTTCCAATCAAAAA
CTCGGCTAGACTTATACTAAGACCAAAGTCTTATCCATTTGTAGATGGAACTTCGACAGCAGCTAGGTCTAGAGGGAAGTTATGAGC
ATTACGTTCATGCATAAC

Figure 11. The *trn*H-*psb*A fragment sequence of UP95

These sequences were uploaded on NCBI by BLASTn to find the differences with other species in Table 7.

Table 7. Some species are homology to UP99 on the *trn*H-*psb*A fragment

Order	Scientific name	Code	Similarity ratio (%)
1	*E. urophylla*	EF507887.1	100
2	*E. grandis*	EF507887.1	99.41
3	*UP95_ E.urophyla x E.pellita*	UP95	100
4	*Syzygium aromaticum*	MH070008.1	85.05

Using Mega X software program we constructed the phylogenetic tree and genetic distances as Fig.12 and Table 8.

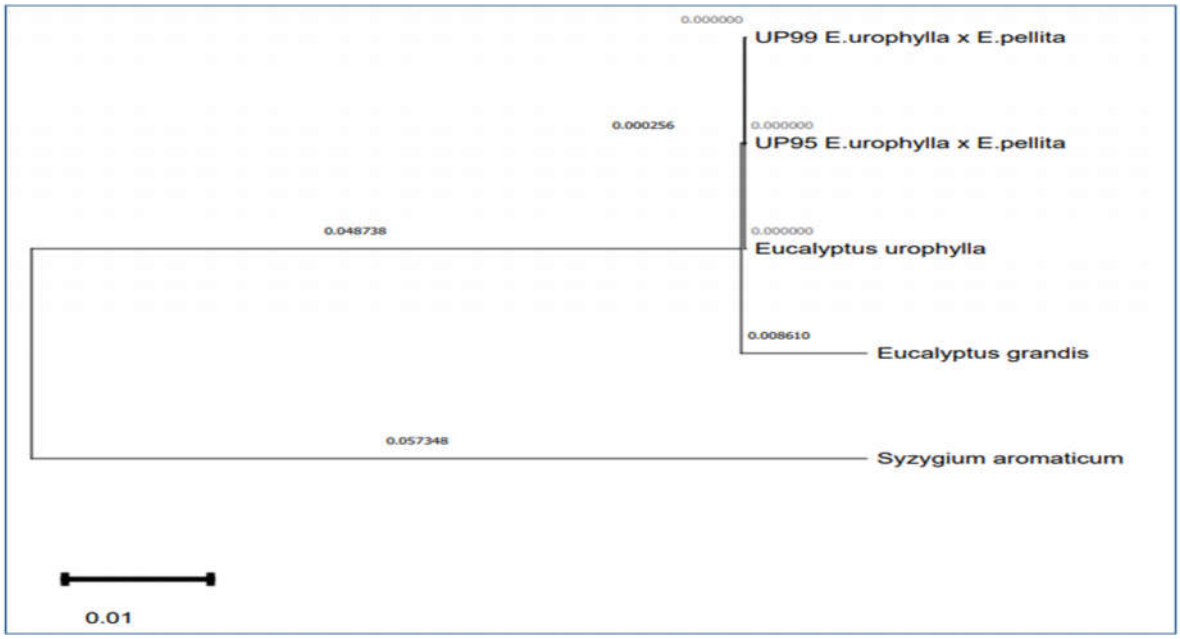

Figure 12. Phylogenetic tree were built based on the *trn*H-*psb*A fragment sequence

Table 8. The genetic distances of UP99 with other species on *trn*H-*psb*A fragment sequence

Scientific name	***UP99_ E.urophyla x E.pellita***	***UP95_ E.urophyla x E.pellita***	***Syzygium paniculatum***	***E. urophylla***	***E. grandis***
UP99_*E.urophyla x E.pellita*					
UP95_ E.urophyla x E.pellita	0.0000				
Syzygium aromaticum	0.1106	0.1106			
E. urophylla	0.0000	0.0000	0.1106		
E. grandis	0.0086	0.0086	0.0966	0.0086	

The results of the phylogenetic tree, genetic distances and similarity ratio showed that there was no difference among UP99, UP95 and *E. urophylla*. And UP99 had a little difference with *E. grandis* (genetic distance was 0.0086). But UP99 have the highest difference with *Syzygium aromaticum* (genetic distance was 0.1106). However, the *trn*H-*psb*A fragment had not yet determined the difference between two hybrid Eucalyptus UP99 and UP95.

3.4. The DNA sequence of *ITS* fragment

The *ITS* fragment sequence of UP99 was 563 bp, the *ITS* fragment sequence of UP95 was 534 bp (Fig.13; Fig.14). There was no difference among three repetitions of UP99 and UP95.

CCGACGTCCCTCTCGACGCGGAGGATCGGGGCTCGGGCACCTCAGGGCGCTCGGCCTTTGTCCTCGGCGGCGCAACGAACCCCGGCG
CGGAATGCGCCAAGGAACTTTAACAAGAGTGCGATGCTCCCGCCGCCCCATACACGGTGCGCGCGCGGGATGCCATGCAATCTCATATTA
GTCATAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAACTGCGATACTTGGTGTGAATTGCAGAATCC
CGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAACCTTTGGTCGAGGGCACGTTTGCCTGGGTGTCACACATGGCGTTGCCCCT
AATCCCCTCCGCCCTCTGAACGGGGCGAGCGGGACTCGGGCGCGTACGATGGCCTCCCGCGACGACCACGTCCCGGTTGGCCCAAAATCG
AGCGTCGGAGCGATCAGCACCACGACATTCGGTGGTTGATTAGACCCCAATGATCAATGTCGCGCGTGCCGCTCATCGCACGCTCCGCGA
ATCTGCTCCTTACCAACGCGACCCCA

Figure 13. The *ITS* fragment sequence of UP99

CCGACGTCCCTCTCGACGCGGAGGATCGGGGCTCGGGCACCTCAGGGCGCTCGGCCTTTGTCCTCGGCGGCGCAACGAACCCCGGCG
CGGAATGCGCCAAGGAACTTTAACAAGAGTGCGATGCTCCCGCCGCCCCATACACGGTGCGCGCGCGGGATGCCATGCAATCTCATATTA
GTCATAACGACTCTCGGCATCGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAACTGCGATACTTGGTGTGAATTGCAGAATCC
CGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAACCTTTGGTCGAGGGCACGTTTGCCTGGGTGTCACACATGGCGTTGCCCCT
AATCCCCTCCGCCCTCTGAACGGGGCGAGCGGGACTCGGGCGCGTACGATGGCCTCCCGCGACGACCACGTCCCGGTTGGCCCAAAATCG
AGCGTCGGAGCGATCAGCACCACGACATTCGGTGGTTGATTAGACCCCAATGATCAATGTCGCGCGTGCCGCTCATCGCACGCTCCGCGA
ATCTGCTCCTTACCAACGCGACCCCA

Figure 14. The *ITS* fragment sequence of UP95

These sequences were uploaded on NCBI to find the differences of UP99 with other species as in Table 9.

Table 9. Some species are homology to UP99 on the *ITS* fragment

Order	Scientific name	Code	Similarity ratio (%)
1	*E. urophylla*	HM596068.1	99.04
2	*E. grandis*	AF058475.1	98.13
3	*E. pellita*	KT631261.1	98.31
4	*UP95_E.urophyla x E.pellita*	UP95	99.81
5	*Syzygium paniculatum*	KM064993.1	90.67

And then the phylogenetic tree and genetic distance were constructed by mega X software as in Fig.15 and Table 10 to find the relationship of UP99 with UP95 and other species.

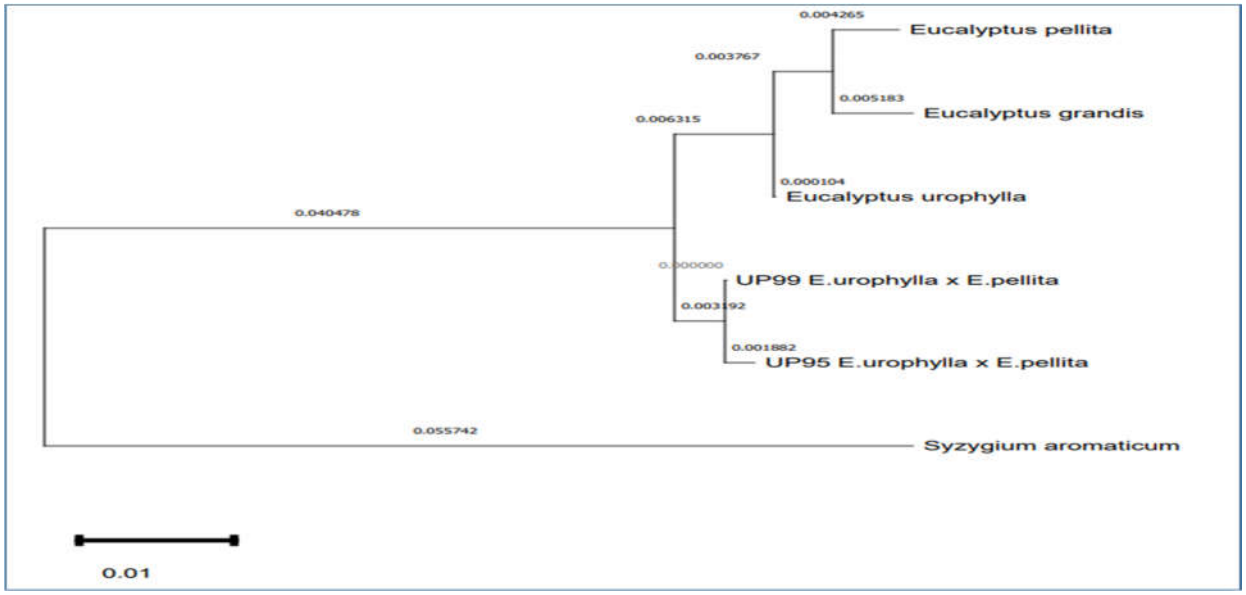

Figure 15. Phylogenetic tree were built based on the *ITS* fragment sequence

Table 10. The genetic distances of UP99 with other species on *ITS* fragment sequence

Scientific name	*UP99_ E.urophyla x E.pellita*	*UP95_ E.urophyla x E.pellita*	*Syzygium paniculatum*	*E. urophylla*	*E. grandis*
UP99_*E.urophyla x E.pellita*					
UP95_ E.urophyla x E.pellita	0.0018				
Syzygium aromaticum	0.0981	0.1004			
E. urophylla	0.0075	0.0094	0.1003		
E. pellita	0.0171	0.0190	0.1091	0.0075	
E. grandis	0.0133	0.0153	0.1077	0.0075	0.0095

The results of the phylogenetic tree combined with genetic distances and similarity ratio indicated that UP99 were 99.81% similar to UP95 (genetic distance was 0.0018), 99.04% similar to *E. urophylla*, 98.31% similar to *E. pellita*, 98.13% similar to *E. grandis*, 90.67% similar to *Syzygium aromaticum*. So, there was the difference among UP99 and UP95 on *ITS* fragment sequence. Therefore, this result suggests that it is better for using *ITS* molecular marker as a DNA barcode to identify bybrid Eucalyptus UP99 and UP95.

3.5. The DNA sequence of *ITS*2 fragment

The results of *ITS* fragment sequence analysis indicated that UP99 was 214 bp, UP95 was 374 bp in the length (Fig.16; Fig.17). There was no difference among three repetitions of UP99 and UP95.

CACATGGCGTTGCCCCTAATCCCCTCCGCCCTCTGAACGGGGCGAGCGGGACTCGGGCGCGTACGATGGCCTCCCGCGACGACC
ACGTCCCGGTTGGCCCAAAATCGAGCGTCGGAGCGATCAGCACCACGACATTCGGTGGTTGATTAGACCCCAATGATCAATGTCGC
GCGTGCCGCTCATCGCACGCTCCGCGAATCTGCTCCTTACCAAC

Figure 16. The *ITS2* fragment sequence of UP99

TGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAACCTTTGGTCGAGGGCAC
GTTTGCCTGGGTGTCACACATGGCGTTGCCCCCAATCCCCTCCGCCCTTTCAACGGGGCGAGCGGGACTCGGGCGCGTACGATGGCCTC
CCGCGACGACCACGTCCCGGTTGGCCCAAAATCGAGCGTCGGAGCGATCAGCACCACGACATTCGGTGGTTGATTAGACCCCAATGAT
CAATGTCGCGCGTGCCGCTCATCGCACGCTCCGCGAATCTGCTCCTTACCAACGCGACCCCAGGTCAAGCGGGGCTACCCGCTGAGTTT
AAGCATATCAATAAGCGGAGGA

Figure 17. The *ITS2* fragment sequence of UP95

These sequences were uploaded on NCBI by BLASTn to find the difference between UP99 and other species in Table 11.

Table 11. Some species are homology to UP99 on the *ITS*2 fragment

Order	Scientific name	Code	Similarity ratio (%)
1	*E. urophylla*	AF390492.1	98.29
2	*E. grandis*	HM596050.1	98.29
3	*E. pellita*	KT631261.1	96.57
4	*UP95_ E. urophyla x E. pellita*	UP95	98.86
5	*Syzygium paniculatum*	AY187204.2	86.78

Using mega X software to construct the phylogenetic tree and genetic distance as in Fig.18 and Table 12.

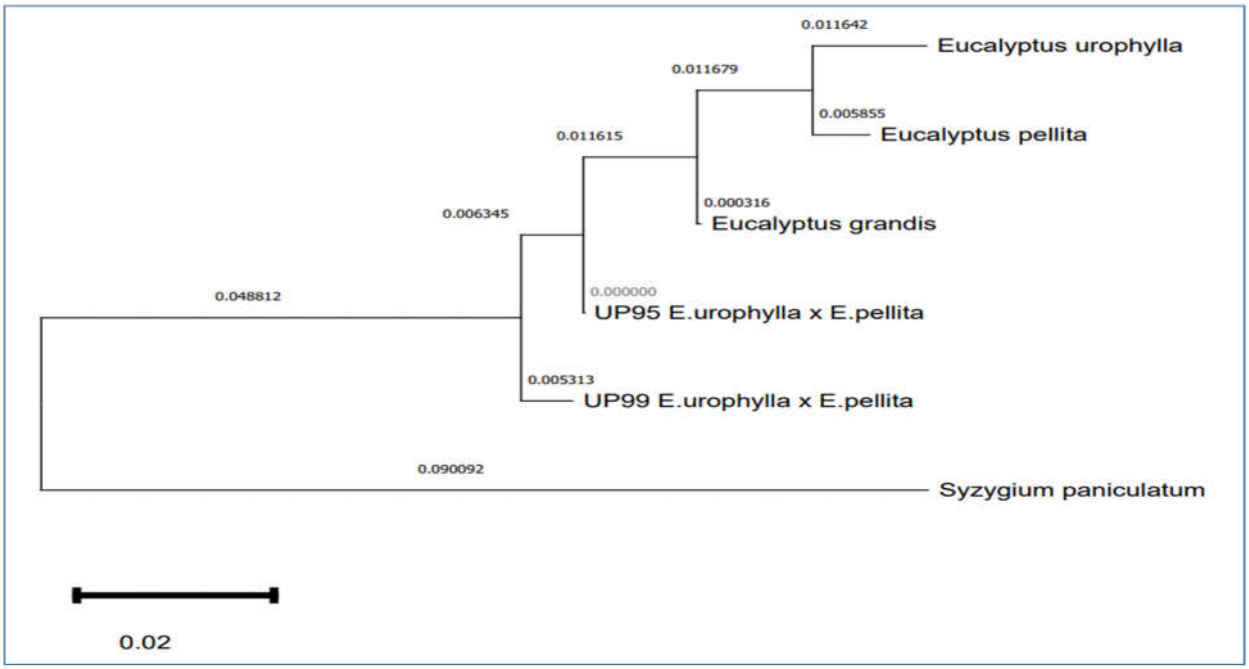

Figure 18. Phylogenetic tree were built based on the *ITS*2 fragment sequence

Table 12. The genetic distances of UP99 with other species on *ITS* fragment sequence

Scientific name	*UP99_ E.urophyla x E.pellita*	*UP95_ E.urophyla x E.pellita*	*Syzygium paniculatum*	*E. urophylla*	*E. grandis*
UP99_*E.urophyla x E.pellita*					
UP95_ E.urophyla x E.pellita	0.0016				
Syzygium aromaticum	0.1477	0.1488			
E. urophylla	0.1740	0.0234	0.1711		
E. pellita	0.0356	0.0297	0.1711	0.0175	
E. grandis	0.0235	0.0116	0.1655	0.0234	0.0175

From the phylogenetic tree based on *ITS*2 fragment sequence combined with genetic distances and similarity ratio indicated that UP99 was 98.86% similar to UP95, 98.29% similar to *E. urophylla* and *E. pellita*, 96.57% similar to *E. grandis*, 86.78% similar to *Syzygium aromaticum*. So, there was the difference between UP99 and UP95 on *ITS*2 fragment sequence. Therefore, it is better for using *ITS*2 molecular marker as a DNA barcode to identify UP99 and UP95.

4. DISCUSSION

Comparing five candidates DNA barcodes (*mat*K, *rbc*L, *trn*H-*psb*A, *ITS* and *ITS*2) between UP99 and UP95 showed that: The *ITS* and *ITS*2 regions were the most efficient DNA barcode sequence with the difference ratio reached 0.19% and 1.4%, respectively (Table 13).

Table 13. Compare five DNA barcodes between UP99 and UP95

DNA barcodes locus	*mat*K	*rbc*L	*trn*H-*psb*A	*ITS*	*ITS*2
Difference nucleotides	0	0	0	1	3
The length	643	687	626	534	214
Difference ratio	0	0	0	0.19	1.4

Some other research before had shown the efficient DNA barcode sequence as In 1999, the study of Steane et al with 35 species Eucalyptus were analyzed by *ITS* sequence [8]. Another study of Fladung had carried out with six chloroplast regions (*rbc*L, *mat*K, *mat*K-*trn*K, *trn*G-*psb*K, *psb*K-*psb*L, *psb*A-*mat*K) and *ITS* region to identified 6 Eucalyptus species in Mexico [3]. The classification based on morphological marker had got a lot of problem for hybrid Eucalyptus species. Therefore, using DNA barcode is a powerful tool complement the morphological method in classification and identification.

5. CONCLUSION

Five candidate DNA barcodes (*mat*K, *rbc*L, *trn*H-*psb*A, *ITS* and *ITS*2) were successfully amplified and sequenced from hybrid Eucalyptus UP99 and UP95. The length of *mat*K, *rbc*L, *trn*H-*psb*A, *ITS* and *ITS*2 fragments were 643, 743-687, 626, 563-534 and 374 for UP99 and UP95, respectively. Comparing these sequences on NCBI indicated that the hybrid Eucalyptus UP99 and UP95 were 100% similarity in *mat*K, *rbc*L, *trn*H-*psb*A fragment sequences; 99.81% similarity in *ITS* fragment sequence; 98.86% similarity in *ITS*2 fragment sequence. The study results showed that among five cadidates DNA barcodes were studied the *ITS* and *ITS*2 regions were the most efficient DNA barcode sequences with maximum genetic distances reached 0.0018 and 0.0016, respectively. These results are an important base for the identification hybrid Eucalyptus UP99 and UP95 for the future development orientations.

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