

Development of DNA Barcodes and Optimization of DNA Isolation and PCR Protocols for a Medicinal Plant *Plectranthus hadiensis*

A.P.P.R. Amarasinghe¹, D.A.S. Siriwardhane², R.R. Samarasekera³, O.V.D.S.J. Weerasena⁴

¹Department of Botany, University of Kelaniya, Sri Lanka

²Sri Lanka Institute of Nanotechnology, Sri Lanka

³Industrial Technology Institute, Sri Lanka

⁴Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka

Abstract: *The medicinal plant Plectranthus hadiensis of Lamiaceae is reported for various biological properties. This plant is substituted in herbal industry by other plants of the same genus due to misidentification. Therefore, the objective of this study was to develop DNA barcodes to discriminate Sri Lankan P. hadiensis from adulterant Plectranthus spp. As DNA barcodes, matK, rbcL and trnH-psbA chloroplast DNA regions were amplified and sequenced. This study also attempted modification of Cetyltrimethylammonium Bromide (CTAB) based DNA isolation and PCR protocols, as genomic DNA from leaves indicated contaminating metabolites that prevented PCR. The barcode sequences resulted in this study were deposited in GenBank nucleotide database. To verify if these barcodes are competent for family or genus assignment of P. hadiensis, the rbcL sequence of P. hadiensis was compared with the rbcL sequences available in the GenBank nucleotide database of the species of the order Lamiales and family Lamiaceae. Comparison of rbcL sequences identified single nucleotide polymorphism among Plectranthus species with a considerable interspecific divergence suggesting that rbcL could serve as a barcode for P. hadiensis.*

Keywords: *Plectranthus hadiensis*, Cetyltrimethylammonium Bromide (CTAB), Polymerase Chain Reaction (PCR), DNA barcodes

1. Introduction

The genus *Plectranthus* of the family Lamiaceae contains about 300 species, distributed in the tropical and subtropical regions of the Old World (Lukhoba, 2006). Only 14 species are recorded in Sri Lanka (Senarathne, 2001) including *Plectranthus hadiensis* (Forssk.) Schweinf. var. *tomentosus* (Benth.) Codd (International Plant Names Index, 2014) which has many applications in the Ayurveda system of medicine (Jayaweera, 1981; Rahayu, 1999). This species is found in the lowlands and lower uplands of Sri Lanka and (Cramer, 1981) and Tamil Nadu, India (Menon and Sasikumar, 2011). Recent studies have shown biological properties of *P. hadiensis* such as cytotoxic, antioxidant, antimicrobial, and anti-inflammatory activities (Gaspar *et al.*, 2002; Laing *et al.*, 2008; Mothana *et al.*, 2010).

In herbal industry *P. hadiensis* is substituted by other plants of the same genus, especially by *Plectranthus barbatus* and *Plectranthus amboinicus* (Lukhoba, 2006; Menon *et al.*, 2011). *P. hadiensis* is difficult to discriminate from the adulterant plants using morphology alone. Besides, substituting one species for another reduces the efficacy of herbal products. Classical plant taxonomy however fails to identify plants in processed products (Frezal and Leblois, 2008). These obstacles can now be overcome by using the sequence of a standard region of DNA (DNA barcoding), as an accurate identification tool (Kress and Erickson, 2008a; CBOL, 2009). It is a promising approach to monitor the adulteration of other herbs in trade and an attractive aid in plant identification (Yao *et al.*, 2009; Srirama *et al.*, 2010; Schori and Showalter, 2011). Therefore, this study was

undertaken with the objective of developing DNA barcodes for *P. hadiensis* from the chloroplast genome, the *psbA-trnH* intergenic spacer, the ribulose-1,4-bisphosphate carboxylase larger subunit gene (*rbcL*) and the ribosomal RNA maturase gene (*matK*).

While developing DNA barcodes, preliminary studies showed that the extracted DNA of metabolite rich *P. hadiensis* using Cetyltrimethylammonium Bromide (CTAB) based Chen and Ronald (1999) or Doyle and Doyle (1990) protocols has inhibited PCR reactions. Thus, modification in CTAB based plant DNA isolation protocol (Chen and Ronald, 1999) was required. Polymerase Chain Reaction (PCR) protocol was also adjusted, as the previous PCR protocols used to amplify barcode regions of plants of Lamiaceae were not successful for *P. hadiensis*. Hence, this investigation also attempted optimizing DNA isolation and PCR protocol that could be useful to barcode *P. hadiensis*.

2. Materials and Methods

2.1. Plant Collection

Plant materials of *Plectranthus hadiensis* for DNA barcoding were collected from the medicinal garden at Bandaranaike Memorial Ayurveda Research Institute (BMARI), Sri Lanka. Specimens were authenticated at the National Herbarium, Sri Lanka and voucher specimens were deposited at herbarium of BMARI (accession number: 1554).

2.2. DNA Isolation

Total genomic DNA was extracted using Cetyltrimethylammonium Bromide (CTAB) DNA isolation protocol (Chen and Ronald, 1999) with modifications. Fresh tender leaves of *P. hadiensis* were washed with deionized water and wiped with 70% ethanol. Glandular hairs of the leaves were removed using sellotapes. 50 mg of leaf tissues were ground to a fine powder in liquid nitrogen in 1.5 mL Eppendorf tube. CTAB buffer was pre-warmed to 65 °C and 700 µL was added (Poly vinyl pyrrolidene-40 (PVP-40) was increased from 2% to 3% in CTAB). After adding 7 µL of 20 µg/mL RNase, the mixture was incubated at 65 °C for 8 minutes. Then 570 µL of chloroform: isoamyl alcohol, 24:1 (CHISAM) (adjusted with phenol as chloroform and isoamyl alcohol mixture: phenol in 1:1 ratio) was added to each sample and the samples were centrifuged at 13000 rpm at 25 °C for 10 minutes. The CHISAM step was repeated twice. The aqueous phase in the supernatant was transferred into a new tube. DNA was precipitated by 0.7 volumes of isopropanol. The mixture was centrifuged at 13000 rpm at room temperature for 5 minutes. The supernatant was removed and the pellet was washed with 70% ethanol, vacuum dried, dissolved in 20 µL of autoclaved ultra-pure water and preserved at -20 °C.

2.3 Quantification and Quality Evaluation of Extracted DNA

The yield of DNA per gram of leaf tissue was measured using a UV spectrophotometer (UV-1700 pharماسpec) at 260 nm. The purity of DNA was determined by the ratio of absorbance at 260 nm to that of 280 nm. The DNA concentration and purity was also determined by running samples on 0.8% agarose gel depending on the intensities of band.

2.4 Optimization of Polymerase Chain Reaction (PCR)

The universal primer pairs (Table 1) were employed for the amplification of the *matK*, *rbcL* and *trnH-psbA* regions of the chloroplast DNA. PCR protocol proposed for plants of Lamiaceae in Theodoris *et al.* (2012) was used as the starting protocol for PCR, however adjusted for MgCl₂ and DNA concentrations (using a concentration series) and annealing temperatures (using a temperature series) of all primers due to unsuccessful amplification of DNA of *P. hadiensis* in the initial protocol. MgCl₂ optimization revealed that 2.5 mM MgCl₂ was ideal for PCR amplification. Additionally, the template DNA concentration of 30 ng per PCR reaction mixture was found optimum.

Table 1: PCR primers used for amplification of the plastid regions

Plastid locus	Primer	Direction	Nucleotide sequence (5' - 3')
<i>rbcL</i>	rbcLa F	forward	ATGTCACCACAAACAGAGACTAAAGC
	rbcLa R	reverse	CTTCTGCTACAAATAAGAATCGATCTC
<i>trnH-psbA</i>	psbA3' f	forward	GTTATGCATGAACGTAATGCTC
	trn Hf_05	reverse	CGCGCATGGTGGATTACAATCC
<i>matK</i>	matk390F	forward	CGATCTATTCAATCAATATTTTC
	matk1326R	reverse	TCTAGCACACGAAAGTCGAAGT

The PCR mixture, with a total volume of 25 µL contained 1x PCR buffer, 0.2 mM dNTPs, 2.5 mM MgCl₂, 0.3 µM each primer, 30 ng of genomic DNA and 1 U Taq DNA polymerase (Gotaq, Promega, USA). PCR was carried out on a Mastercycler (Eppendorf) thermocycler as: initial denaturation at 94 °C for 4 min then 35 cycles of DNA denaturation at 94 °C for 30 s, annealed at 47 °C for 1 min (*matK* and *psbA-trnH*) and at 53 °C for 1 min (*rbcL*), extension at 72 °C for 1 min followed by final extension step at 72 °C for 4 min. PCR products were run on ethidium bromide stained 1% agarose gel with 100 bp DNA ladder to visually estimate the fragment sizes relative to the ladder marker.

2.5 DNA Sequencing and Data Analysis

The PCR products were purified by Illustra GFX PCR and Gel Band Purification kit (GE Healthcare UK Limited, UK). The cycle sequencing reactions of the purified fragments were performed using the BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems, USA) according to the manufacturer's instructions. Cycle sequencing temperature profile was 30 cycles of 95 °C 15 s (50 °C 15 s for *psbA-trnH*, 52 °C 15 s for *matK*, *rbcL*) and 60 °C 1 min. Products were purified by ethanol precipitation as indicated in manufacturer's instructions and run on ABI 3500 Genetic

Analyzer sequencer (Applied Biosystems, USA). Sequencing was repeated three times with the same primers.

All sequences have been deposited in GenBank nucleotide database after analyzing with BioEdit 7.1.3.0 sequence alignment software. The *rbcL* sequence of *P. hadiensis* was compared with that of four *Plectranthus* spp. with ClustalW multiple alignment tool. To verify if these barcodes are competent for family or genus assignment in authenticating *Plectranthus* genus, the *rbcL* sequences of the order Lamiales (2-5 species per family) were downloaded from GenBank. Moreover, the *rbcL* reference sequences of two species the family Lamiaceae were used to test whether these sequences could be aligned among the different genera. The interspecific sequence divergence of *P. hadiensis* and the other *Plectranthus* spp. were estimated using the program MEGA 6.0 (Tamura *et al.*, 2013) with Kimura-2-Parameter (K-2-P) model. Based on the K-2-P distances, a Neighbor-Joining (NJ) tree was constructed.

3. Results and Discussion

3.1. DNA Isolation

The ratio of absorbance values at 280 nm and 260 nm (A260/280) ranged 1.5-1.7 when DNA was isolated from Chen and Ronald (1999) and Doyle and Doyle (1990)

protocols. Their PCR was also unsuccessful may be due to PCR inhibiting contaminants of extracted DNA such as proteins or polyphenolic compounds. Hence, Chen and Ronald (1999) DNA extraction protocol was optimized to remove proteins and polyphenols. As some diterpinoids are associated with glandular hairs of *P. hadiensis* (Betty and Thoppil, 2008), these hairs were removed from the leaves before DNA isolation. To remove polyphenol compounds 2% w/v PVP was increased till 3%. For protein precipitation, phenol was introduced to CHISM repeating this step. Although additional steps reduced DNA yield, the quality has improved showing A260/280 from 1.8-2.0, and resulting PCR bands.

3.2. Optimization of Polymerase Chain Reaction (PCR)

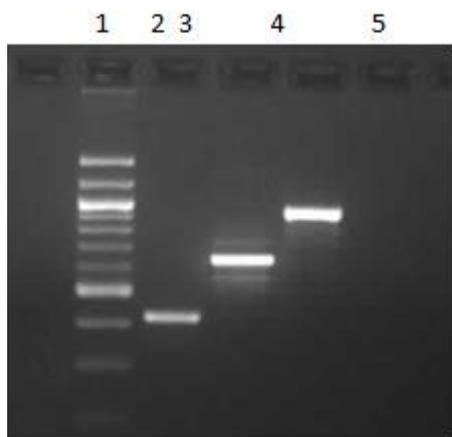


Figure 1: PCR amplified products: Lane 1- 100 bp DNA ladder, Lane 2- PCR product of *psbA-trnH*, Lane 3- PCR product of *rbcL*, Lane 4- PCR product of *matK*, Lane 5- negative control

The optimized PCR protocol resulted successful amplification of selected barcode regions, and as shown in Figure 1. PCR products were in the expected sizes such as; *psbA-trnH* 300-800 bp, *rbcL* 500-800 bp and *matK* 800-1500 bp (Kress and Erickson, 2008b).

3.3. DNA Sequencing and Data Analysis

The lengths and accession numbers of *psbA-trnH*, *rbcL* and *matK* sequences of *P. hadiensis* deposited in the GenBank nucleotide database are listed in Table 2.

Table 2: Lengths and GenBank accession numbers of *psbA-trnH*, *rbcL* and *matK* of *Plectranthus hadiensis*

Plastid locus	Length (bp)	Accession No.
<i>psbA-trnH</i>	346	KC146391
<i>rbcL</i>	601	KC211322
<i>matK</i>	643	KC211323

Among the used barcode regions, only *rbcL* reference sequences of the other *Plectranthus* spp. were recorded in GenBank to date. Thus, *rbcL* regions of *P. hadiensis* was compared with reference sequences of *P. amboinicus* (KJ372411), *P. barbatus* (U28882), *P. amicum* (KF496704) and *P. caninus* (JQ072901). A total of 14 single nucleotide polymorphisms (SNPs) were detected at the aligned positions 138, 163, 164, 177, 181, 195, 255, 262, 271, 272, 415, 416, 424 and 438 of five *Plectranthus* species (Table 3).

Table 3: Single Nucleotide Polymorphisms of *rbcL* of *Plectranthus hadiensis* and other *Plectranthus* spp.

Species	138	163	164	177	181	195	255	262	271	272	415	416	424	438
<i>P. hadiensis</i>	A	G	C	C	C	C	G	G	G	C	G	T	G	G
<i>P. amboinicus</i>	G	G	C	T	T	T	C	C	A	A	A	C	A	A
<i>P. barbatus</i>	G	C	G	T	T	T	G	G	A	A	G	T	G	A
<i>P. amicum</i>	G	G	C	T	T	T	C	C	A	A	A	C	A	A
<i>P. caninus</i>	G	G	C	T	T	T	G	G	A	A	G	T	G	A

The Neighbor-Joining (NJ) tree constructed based on the K-2-P distances from the *rbcL* sequences of the order Lamiales is shown in Figure 2. The sequence divergences among *P. hadiensis* and other *Plectranthus* spp. were considerable with the average interspecific distance of 0.9%. Together with other species from the order Lamiales, the NJ tree of

rbcL DNA sequences correctly placed *P. hadiensis* and other *Plectranthus* spp. with the family Lamiaceae and the genus *Plectranthus* with bootstrap percentages of 64% and 83%, respectively (represented by dots at the nodes of the branches).

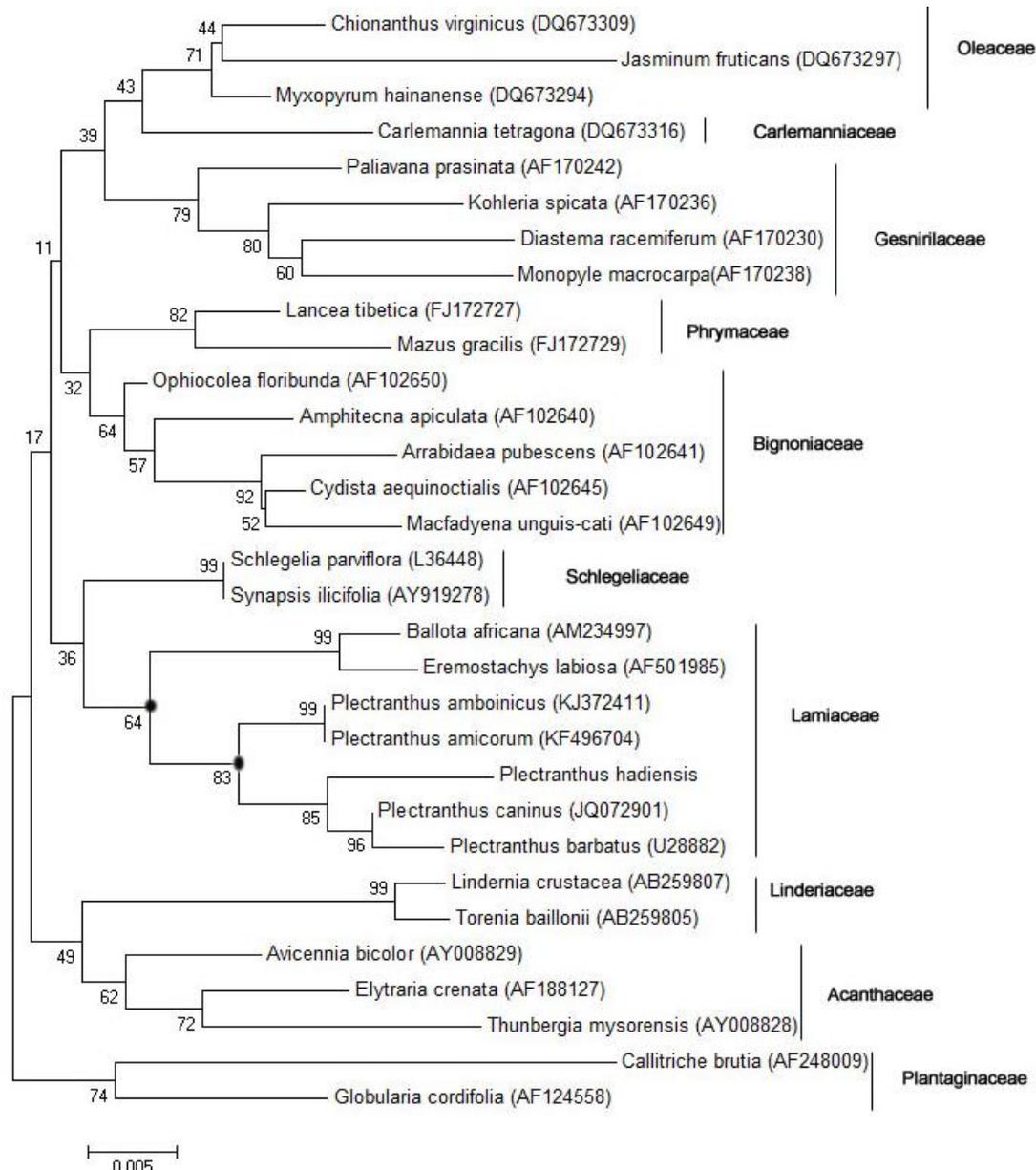


Figure 2: The NJ Tree constructed from the *rbcL* sequences of the order Lamiales based on the K-2-P distances. Bootstrap analysis (1000 replicates) was conducted to estimate the statistical supports of the topology of the consensus tree. The bootstrap percentages are shown at the branches. GenBank accession numbers are in parentheses.

According to Kress and Erickson (2008a) a nucleotide sequence must satisfy three criteria to be practical as a DNA barcode such as the sequence should contain a sufficient variation to discriminate between species, consist of flanking sites to develop universal PCR primers and have a short sequence length to facilitate current capabilities of DNA extraction and amplification. As the *rbcL* region was successfully amplified and sequenced placing the species in the correct family and genus and showed considerable interspecific divergence, this region can be considered as a suitable barcode to discriminate *P. hadiensis* from some other species of the same genus.

To find out the best barcode region to identify *P. hadiensis* from other *Plectranthus* spp. *psbA-trnH* and *matK* also should be compared using the same approach once the sequences are available. Further, DNA barcoding should also be carried out for the commercial *P. hadiensis* herbal preparations to find the most effective and accurate DNA

barcodes for the species identification after processing the plant.

4. Conclusion

Optimized DNA isolation protocol and PCR protocol can be used for successful amplification of barcode regions of metabolite rich *P. hadiensis*. Comparison of *rbcL* sequence of *P. hadiensis* with reference *rbcL* sequences of other *Plectranthus* spp. identified single nucleotide polymorphism. Additionally, *rbcL* region placed the species in the correct family and genus showing a considerable interspecific divergence.

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