



Research Article – Molecular Biology

Optimizing the pure genomic DNA isolation procedure for *Plectranthus amboinicus* DNA – A prerequisite for further genomic studies

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Abstract

The DNA isolation procedure for different plant groups have been studied and standardized. The isolation of pure genomic DNA is the most essential component for any type of molecular studies. The present work is aimed to identify suitable DNA markers for the amplification of *P. amboinicus* DNA becomes a great hurdle for DNA barcoding studies carried out by *rbcL* and *matK* primers Used in the members of Lamiaceae. To solve this problem, The DNA was extracted by three methods from fresh young leaf tissue of *P. amboinicus*. After the evaluation the outcome of these methods, one most suitable modified method was selected for isolating DNA from young leaves of *P. amboinicus* and selected for suitable DNA barcoding markers for PCR amplification. The quality and quantity of DNAs are a prerequisite for genetic studies for a variety of plants including *P. amboinicus*. The quantity and quality of the DNA extracted by this method was used for suitable DNA barcoding markers selection.

Key words: *P. amboinicus*, DNA Isolation, Comparative analysis, PCR, DNA Barcoding

Introduction

The isolation of high quality and quantity molecular mass pure genomic DNA is essential for many molecular biology applications. Many protocols are available for the extraction of DNA from plant material. Medicinal and Aromatic plants (MAPs) utilization and conservation has attracted global attention. Several of these MAP's have high amounts of polysaccharides, polyphenols, tannins, hydrocolloids (Sugars & Carragenans) and other secondary metabolites such as alkaloids, flavonoids, phenols, terpenes and quinines which would interfere with the DNA isolation procedures. Especially The members of lamiaceae family contains high phenolic content together with certain polysaccharide content together with certain polysaccharide content makes the isolation of high quality pure genomic DNA problematic.

Plectranthus amboinicus Lour. (Lamiaceae), syn. *Coleus aromaticus* (Benth.) is commonly known as Indian/country borage. It is a large succulent herb with aromatic leaves, found abundantly in India. The leaves of this plant are traditionally used for the treatment of severe bronchitis, asthma, diarrhea, epilepsy, renal and vesicle calculi and fever.¹ *C. aromaticus* has been reported to exhibit antilithiotic,² chemo preventive,³ antiepileptic and antioxidant properties.

Plants represent a more complex barcoding problem than other eukaryotes (such as animals) because plant mitochondrial genomes have a low nucleotide substitution rate⁴. It has been found that genes other than COI should be used for plant identification because there are not enough changes in the COI between 15 different plant groups. Various combinations of plant specific markers (*rpoC1* + *rpoB* + *matK* or *rpoC1* + *matK* + *trnH-psbA*; *rbcL* + *trnH-psbA*; *atpF-H* + *psbKI* + *matK*) can be used for plant barcoding. The current literature seems to be coming to the conclusion that two or more markers are needed to identify plants. There is still a great deal of research that

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needs to be done to create markers that can reliability analyze divergent taxonomic groups.

Several genera currently recognized within Ocimeae, such as *Plectranthus*, have no clear synapomorphies and could be polyphyletic. Although a few molecular phylogenetic trees of Lamiaceae and subfamily Nepetoideae have been produced using plastid DNA restriction site data⁵, *rbcL*,⁶ *rbcL* and *ndhF*,⁷ none deal in any detail with Ocimeae. Even in the cases of *Ocimum* and *Platostoma* where morphological parsimony analyses have been published,^{8,9} nodes lack statistical support. Robust phylogenetic analyses are much needed in order to efficiently explore the medicinal and economic uses of the group.

Problems arising of DNA isolation from the species of *Plectranthus*

The problems encountered in the isolation and purification of DNA specially from the species of *Plectranthus* include degradation of DNA due to endonucleases, co isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols, other secondary metabolites which directly or indirectly interfere with the enzymatic reactions. Moreover, the contaminating RNA that precipitates along with DNA causes many problems including suppression of PCR amplification¹⁰, interfere with DNA amplification involving random primers¹¹ and improper priming of DNA templates during thermal cycler sequencing.

Different plant taxa often may not permit optimal DNA yields from one isolation protocol. For example, some closely related species of the same genus require different isolation protocols. Thus, an efficient protocol for the isolation of pure form of DNA is needed which often must for further molecular studies. Various protocols for DNA extraction have been successfully applied to many plant species,¹² which were further modified to provide pure form of DNA suitable for several kinds of analyses.¹³ We have tested previously established DNA isolation protocols but these methods resulted in DNA with lot of impurities and not very suitable for DNA Barcoding analysis.

Modifications were made to minimize polysaccharides co-isolation and to simplify the procedure for processing large number of sample. The protocol optimized for DNA Barcoding proved to be inexpensive with relation to the use of Primer,

quality of DNA, usage of dNTPs, Taq Polymerase and the reaction volume. Thus the protocol derived for both genomic DNA isolation and DNA Barcoding is genus independent efficient, inexpensive, simple, rapid and yields pure DNA amplifiable by PCR as indicated by the results of the DNA Barcoding technique. The isolated DNA would be suitable for further PCR downstream application.

To come over this problem, the DNA isolation methods need to be modified to each plant species and even to each plant tissue because of the presence of the various secondary metabolites. The published procedures tested were inadequate for the extraction of high quality of DNA and which combinations of barcoding markers were amplified in *Plectranthus amboinicus* isolated DNA with agarose gel electrophoresis of PCR amplified products.

Materials and Methods

Plant Material

The samples of young and tender leaves of *P. amboinicus* were collected from Tanjore, Tamil Nadu. After washing the plant tissue with sterile water and subsequently with 70 per cent alcohol, 1g of fresh leaf tissue of species was taken and then it was chopped into fine pieces and subjected to genomic DNA isolation. Genomic DNA was extracted from fresh leaves by adopting the modified CTAB method¹² outlined by without using liquid nitrogen. Genomic DNA yield was expressed as µg DNA per mg of leaves tissue.

Testing DNA Isolation methods

Method 1: HipurA Plant Genomic DNA extraction Kit (Himedia, Cat#MB502-PR)

Method 2: Described by Doyle and Doyle 1987. Modified Cetyl Trimethyl Ammonium Bromide (CTAB) extraction protocol was used which was applied in many plant species. Extraction buffer in this protocol was included: 2% CTAB, 100 mM TrisHCl, 2% PVP, 1.4M NaCl, 20 mM EDTA (pH: 8.0), 2% β-mercapto ethanol, Chloroform: Isoamyl alcohol (24:1), Isopropanol, RNase, 70% Ethanol, TE buffer (10mM TrisHCl, 1mM EDTA (pH: 8.0), immediately prior to use.

Method 3: Described by Sunil Kumar *et al.* 2012. Suspension buffer (pH 8.0) in this protocol was included: 50 mM EDTA, 120 mM TrisHCl, 1M NaCl,

0.5 M Sucrose, 2% Triton-X 100 and 0.2% β mercapto ethanol (to be freshly added just before use), High salt TE buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH: 8.0).

Suspension buffer solution and TE buffer for modified DNA extraction Sunil Kumar *et al.* 2012 were prepared according to Table 1.

Table 1. Different Concentration of Modified method of Sunil Kumar *et al.* (2012)

S. No.	Name of the Solutions	Standard Concentration	Modified Concentration
Suspension Buffer			
1	EDTA	50mM	20mM
	Tris -HCl	120mM	100mM
	NaCl	1M	1.5M
	Sucrose	0.5M	1M
	Triton-X 100	2%	2.5%
2	B-mercapto ethanol	0.2%	1.5%
	High Salt TE Buffer		
	NaCl	0.5M	1M
	Tris-HCl	10mM	20mM
	EDTA	1mM	2mM

Modified DNA extraction method of Sunil Kumar et al. 2012 was as follow

(1) Preheat suspension buffer in water bath at 65°C. Grind 1g of young leaves of *P. amboinicus* to fine powder in ice cold condition in the presence of 100mg PVP (Poly Vinyl Pyrrolidone) by using pre chilled mortar and pestle (-40°C/-80°C) (2) Transfer the content to 2 mL micro centrifuge tubes and suspend in two volumes of suspension buffer (3) Invert and mix gently and incubate at 60°C for 30 min (4) Centrifuge the suspension at 10,000 rpm for 10 min at room temperature. (5) Add 1.5 mL of extraction buffer and incubate at 60°C for 30 min (6) Centrifuge at 10,000 rpm for 10 min at room temperature (7) Carefully transfer the aqueous phase into a new tube (8) Add double volume of chloroform : Isoamyl alcohol (24:1) and invert gently 10 to 15 times and centrifuge at 10,000 rpm for 10 min (9) Add double volume of chilled isopropanol and keep at -20°C for one hour to precipitate the DNA (10) Centrifuge at 10,000 rpm for 10 min and discard the supernatant (11) To the pellet, add 70% chilled ethanol and spool out the pellet carefully and centrifuge again at 10,000 rpm for 10 min (12) Discard the supernatant

and vacuum dry or air dry the pellet at room temperature (13) Add 100 μ l of high salt TE buffer (14) Add 3 μ l RNase and keep at 37°C for 30 min (15) Add 3 M Sodium acetate (16) Spool out the DNA, wash in 70% ethanol, air or vacuum dry (17) Add 30 to 50 μ l (depending upon the pellet) of TE buffer to dissolve the precipitate (18) Store at -20°C/-40°C till further use.

Qualitative and Quantitative analysis of Extracted DNA

The yield of DNA per gram of fresh leaf tissue extracted was measured using a Nano photometer (Implen, P360 Version 1.2.0) at 260nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples in 0.8% agarose gel based on the intensities of band when compared with the 1 kb DNA marker (Used to determine the concentration).

Optimization of PCR based DNA Barcoding Studies

The PCR reactions contain totally 35 cycles and each 25 μ l reaction volume containing 10mM TrisHCl (pH 8.3), 2.5 mM MgCl₂, 25 mM dNTPs mix, 0.2 μ M of each forward and reverse primers in various combinations, 10x Taq buffer, 1U of Taq DNA Polymerase and 50 ng of template DNA. The DNA barcoding programme was performed with matK-1 (F:CCTATCCATCTGGAAATCTTAG, R:GTTCTAGCACAAAGAAAGTCG), matK-2(F:CGATCTATTCATTCAATATTTC, R:TCTAGC ACACGAAAGTCGAAGT) and rbcL-1 (F: ATG TCACCACAAACAGAGACTAAAGC, R: GTAA AATCAAGTCCACCRCG) rbcL-2 (F:ATGTCA CCACAAACAGAAAC, R: TCGCATGTACCTG CAGTAGC) primers in different combination of forward and reverse primers for denaturation 94°C for 45 sec, annealing at 48°C for 30 sec and extension 72°C for 1min. The final extension was carried out at the same temperature for 10 min and the hold temperature of 4°C at the end. The PCR amplified products were electrophoresed on 1.8% (w/v) agarose gels, in 1x TAE buffer at 60 V for 3 hrs and then stained with ethidium bromide

(0.5 µg/ml). Gels with amplification fragments were visualized and Photographed under UV gel documentation system (Alpha Innotech). 1 kb DNA was used as molecular marker (GeNei, Bangalore) to know the size of the fragments.

Results and Discussion

Choice of the material

Proper choice of the plant tissue is very important for DNA extraction.¹⁴ In the present work, young leaf tissue was harvested from two-week old *P. amboinicus* used for DNA extraction since young leaf contain low polyphenolic and terpenoid compounds then older tissue.¹⁵

DNA yield

Yield of the DNA extracted by three methods were listed in Table 2. The DNA yield by modified method¹³ was significantly higher than those obtained by HipurA kit method, and standard CTAB method. DNA extracted by using the method of Doyle & Doyle had not yield good quantity and quality from fresh leaves of *P. amboinicus*. In our research, a high yield of DNA was obtained from both fresh and dry leaf tissues of *P. amboinicus* using modified method¹³. Probably because the young *P. amboinicus* leaves contain less secondary metabolites.

Table 2. Comparison of Quality and Quantity of genomic DNA isolated from fresh leaves of *P. amboinicus*

S. No	Methods	Fresh leaves	
		DNA yield (µg/g)	A260/A280
1	Commercial Kit	1.57±0.04	1.45±0.07
2	Standard CTAB	22.14±1.85	1.72±0.03
3	Sunil Kumar <i>et al</i> Method	89.13±2.81	1.93±0.07

The results are mean of triplicates determination ± standard deviation. Data are means ± SD (n=3)

The lowest DNA yield was obtained by the method reported by Doyle & Doyle¹² and HipurA kit method. This result accorded with.^{16,17,18} By using Doyle & Doyle, Ostrowska 1998 yield was 48-67 µg per gram (equal to 4.8-6.7 µg per 100 mg) DNA from *Pinus radiata*, Abu-Rromman got the lowest DNA yield and poor quality from Sage (*Salvia officinalis*) and yield was DNA obtained from *Plectranthus forskohlii*.¹¹

Table 3. Details of Barcoding Primers using PCR analysis on DNA from *P. amboinicus*

S. No	Primers	Sequences
1	matK-1	Forward: CCTATCCATCTGGAAATCTTAG
		Reverse: GTTCTAGCACAAGAAAGTCG
2	matK-2	Forward: CGATCTATTCATTCAATATTTTC
		Reverse: TCTAGCACACGAAAGTCGAAGT
3	rbcL-1	Forward: ATGTCACCACAAACAGAGACTA
		AAGC Reverse: GTAAAATCAAGTCCACCRCG
4	rbcL-2	Forward: ATGTCACCACAAACAGAAAC
		Reverse: TCGCATGTACCTGCAGTAGC

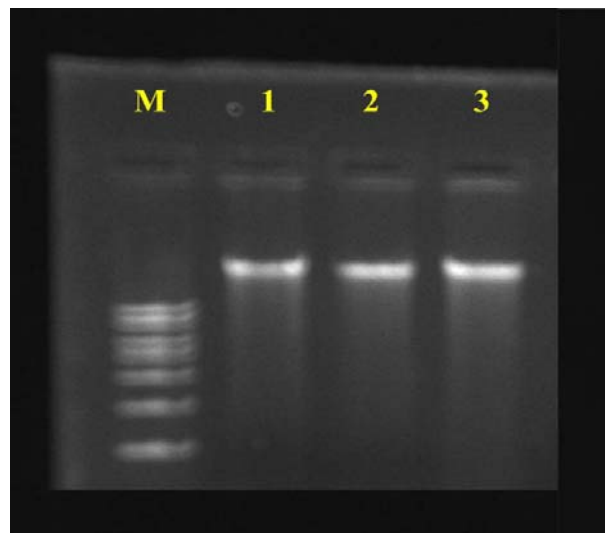
Purity

The assessment of the purity of nucleic acid sample is often performed by a procedure commonly referred to as the OD_{260/280} ratio. Although this procedure was first described by Warburg & Christian¹⁹ as a means to measure protein purity in the presence of nucleic acid contamination, it is most commonly used today to assess purity of nucleic acid sample.²⁰ A pure sample of DNA has the ratio at 1.8±0.2.²¹

The mean OD_{260/280} ratios obtained to DNA extracted by these three methods HipurA kit, CTAB and Sunilkumar *et al*, were higher than 1.9. In these three methods RNA disposal was not involved, hence there existed some RNA residues, as determined by the electrophoresis on agarose gel (Fig. 1 there were clear main bands observed). The mean OD_{260/280} ratios of CTAB method, HipurA kit method were observed in the ratio between 1.4 to 1.7. It means the extracted DNA was relatively free from RNA and Protein contamination. RNase was used to remove RNA from DNA in all the three procedure.

Integrity

The integrity presence of high molecular genomic DNA was determined by electrophoresis 0.8% agarose gel. High molecular DNA bands were obtained from all these three methods while showed bands with smear with the bottom of the lane 1,2,3 (Fig. 2), demonstrating that the DNA were intact but there existed some RNA or Protein residues.



Genomic DNA resolved on 0.8% agarose

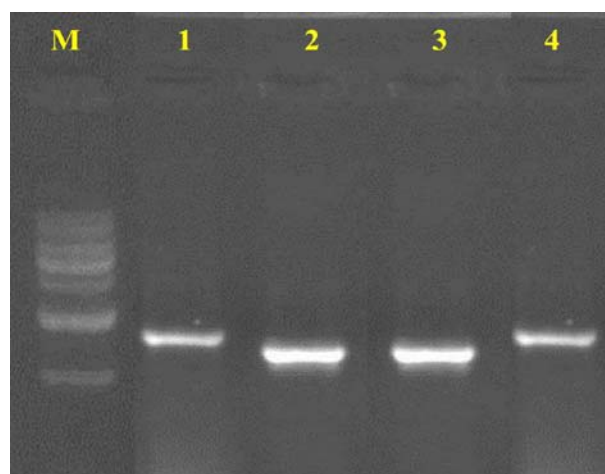
M-1Kb DNA Marker

1-HipurA Kit Method

2-Standard CTAB Method

3-Modified Sunil Kumar *et al* Method

Fig. 1. Genomic DNA of *P. amboinicus* resolved in 0.8% of Agarose Gel Electrophoresis



M- 1 kb DNA ladder; 1-matK-1 F+R; 2- matK-2 F+R; 3- rbcL-1 F+R; 4- rbcL-2 F+R

Fig. 2. Polymerase chain reaction using different combinations of rbcL and matK barcoding primers on DNA from leaves samples of *P. amboinicus* by using method 3

Functionality

There are at least three main contaminants associated with plant DNA, Polyphenolic compounds, polysaccharides and RNA.²² Polysaccharides, which

are difficult to separate from DNA,²³ interfere with several biological enzymes such as polymerases, ligases and restriction endonucleases.²⁴ More over found that when polysaccharides were not removed, the DNA would not amplify in PCR reaction.¹⁴

There are two different viewpoints on the effect of RNA residue. Some researchers hold the opinion that contaminants like RNA often inhibit restriction endonucleases digestion and/or PCR amplification.^{25,26,27} There is also new data indicating that RNA contamination can reduce the effectiveness of many enzymatic processes.^{28,29,30} Furthermore, the RNA degrades at high temperature in the presence of magnesium ions and the release nucleotide incorporation in the PCR condition. While other argues that the presence of the RNA in DNA extracted is not major problem as this usually does not interfere with PCR or restriction digestion.^{31,32} Because RNA is, by nature, transient and unstable unlike DNA. RNA is ubiquitously degraded with striking efficiency in all cells.³³ Much of the RNA is cut by ribonucleases or RNases that are released when the cells are broken open and the rest will not last in an environment outside the cell and will degrade anyways even without RNase.

The PCR reaction using DNA barcoding studies, the different types of primers used for PCR amplification process. The isolated DNA was amplified with both rbcL and matK primer combinations. Based on the obtained result, strongly recommended that the rbcL and MatK primers were amplified with isolated DNA from *P.amboinicus* through PCR amplification. Our result will help to those are all doing barcoding research in the members of Lamiaceae especially the species of *Plectranthus*.

Conclusion

In this study, three methods were used for isolating DNA from *P. amboinicus* were compared and analysed from the following perspectives: yield of DNA, the purity of DNA acquired, intactness, and functionality. All the three methods compared in this study turned out to be suitable to extract DNA from *P. amboinicus*. In summary, the conclusions in this research are as follows:-

1. The yield of DNA from *P.amboinicus* by Sunil Kumar *et al.* 2012 method are significantly

higher than those obtained by the CTAB and HipurA kit method.

2. The extraction method had a significant effect on the DNA yield and OD_{260/280} ratio, fresh leaf tissue of *P. amboinicus*.
3. After evaluating the yield, purity, integrity, functionality among the three methods, the Sunil Kumar *et al.* method was considered an ideal protocol to isolate DNA from *P. amboinicus* by using fresh leaves.
4. Besides, the quality and quantity of the DNA extracted by this method were high enough to perform hundreds of PCR-based DNA barcoding studies by using of *rbcl* and *matK* primers.

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