International Journal of Molecular Biology and Biochemistry
www.biologyjournals.net
Online ISSN: 2664-651X; Print ISSN: 2664-6501
Received: 02-11-2018; Accepted: 04-12-2018; Published: 02-01-2019
Volume 1; Issue 1; 2019; Page No. 04-08

Developing a protocol for the isolation of high quality DNA from carbohydrate rich medicinal plant for molecular identification

Laxmi Kant Pandey[1], Satya Prakash Tantuway[2], Yasmin Bano[3], Nitin Swamy[4], Ranjan Singh[5*], Sundaram Bhuvaneswari[6]
[1-4, 6] Department of Biotechnology, St. Aloysius College, Jabalpur, Madhya Pradesh, India
[5] Department of Biotechnology, Choithram college of Professional Studies, Madhya Pradesh, India

DOI: https://doi.org/10.33545/26646501.2019.v1.i1a.2

Abstract
There is an increasing international market for medicinal plants, which are used both for herbal medicine and for pharmaceutical products. For rapid and accurate identification of plants DNA barcoding is most authentic tool. The aim of this study is first optimize a method for the genomic DNA isolation from non-identified, carbohydrate rich, plant leaf tissues. Than do the DNA barcoding by using the ITS 2 as a potential molecular marker. In this study we first optimize a method for the genomic DNA isolation from non identified, carbohydrate rich, plant leaf tissues. For the optimization of DNA isolation method different combination and permutation were done at different stages of the DNA isolation. Further, the genomic DNA was utilized as a template for the amplification and sequencing of ITS2 region. BLAST search was performed against GenBank database by using annoted ITS2 region as sample query. We found very high yield around 123 µg per gram of leaf samples and the ratio at 260/280 nm found to be 1.82. Hence the yield as well as quality of the DNA was very good. Two PCR products of 419 and 390 bp were amplified by using ITS2F1, ITS2F2 as forward and ITS2R as reverse primers respectively. In annotation out of 419, 228 bp sequences were annotated as ITS2 for this plant in blast search it shows close resemblance to the plants of plantaginaceae in the order lamiales. In herbarium identification the plant was identified as *Anisochilus carnosus* (Lf) which belongs to Lamiaceae family in the order lamiales.

Keywords: PCR, ITS2, DNA barcoding, Sequencing

Introduction
The World Health Organization estimates that 80 percent of the world's population utilizes traditional medicines for healing and curing diseases [1]. The sudden increase in exports of medicinal plants in the past decade testifies to the worldwide interest in these products as well as in traditional health systems [2]. Indian medicinal plants are the essence of ayurveda and ayurvedic treatments. There are more than 600 plants have been listed in the encyclopedia of ayurvedic medicinal plants of India**.** There is an increasing international market for medicinal plants, which are used both for herbal medicine and for pharmaceutical products. Medicinal plants cover a wide range of plant taxa and closely related species. Accurate and rapid authentication of these medicinal plants and their adulterants is very tough to do at the scale of international trade in medicinal plants. For rapid and accurate identification of plants DNA barcoding is most authentic tool. The term ''DNA barcode'' for global species identification was first coined by Hebert in 2003 [3, 4] and has gained worldwide attention in the scientific community [5, 6, 7, 8, 9] DNA barcoding used in various biological applications includes the discovery of cryptic species [10] for reconstructing food webs [11] and medicinal plants identification in mixtures [2]. Several DNA regions, including atpF– atpH, matK, rbcL, ndhj, ycf5, accD, rpoB, rpoC1, psbK–psbI, trnH–psbA, trnL-F, and ITS as well as their combinations, have been used to provide a standard plant barcode [13, 20].

Compared with whole ITS region, ITS2 was more suitable for species identification because of its short length, and high efficiency for PCR amplification [22, 23]. ITS2 because of its shorter sequence have high recovery rate from plant materials found within herbal products. In addition, the secondary structures of ITS2 sequences could be used as molecular morphological characteristics for species identification [24, 25]. It has been proposed that the ITS2 sub-region should be the standard molecular marker for species authentication and for plant phylogenetic analysis [22, 25].

The faith of the consumers is decline towards the use of herbal drugs due to the prevailing trend of adulteration and substitution [26]. Substitution of main herbal ingredients by some other species used in herbal products result in reduced therapeutic potential of the original drug, posing a serious threat to the health of the consumers [27]. The diagnostic morphological features of the plant species on which the traditional taxonomic system is based cannot be used for identifying powdered or otherwise processed plant materials. Thus, there is an urgent need to have in place broadly acceptable commercial tools for detecting substitution and authentication of herbs used industrially. In this study, we try to authenticate ITS2 region as DNA barcode for unknown plant which are used for treatment of paralysis by the local tribes.

Material and Methods
Collection and morphological identification of plant sample
The plant sample was collected from village Paderiya Kalan, Shahpura block, Dindori district, Madhya Pradesh, India. The location of the site was recorded in Google map. The plant habitat, hight, leaves colour, leaves shape and phyllotaxy recorded. The whole plant with roots was taken out and washed

with water to remove any kind of contamination. Colour of roots and stem were noted down. Fresh young leaves for DNA isolation were plucked by forceps, washed and kept into a glass bottle containing absolute ethanol and cap closed tightly. For morphological identification herbarium voucher sheet was prepared. The herbarium of the plant was sent to the Botany Department of State Forest Research Institute (SFRI) Jabalpur, Madhya Pradesh, India for its morphological identification.

DNA Extraction, Amplification, and Sequencing

The isolation of genomic DNA was tried from different types plant leaves i.e. young fresh, dry and ethanol preserved leaves. Initially CTAB based extraction buffer [28] was employed for isolating genomic DNA. To get the optimal result several modifications has been made to the existing methodology for genomic DNA isolation. The optimized sop was used to isolate the genomic DNA from leaf sample. The ITS2 sequences were amplified using the following pair of universal primers ITS2-F, 5′-YGACTCTCGGCAACGGATA -3′ and ITS2-R, 5′-RGTTTCTTTTCCTCCGCTTA -3′ [29]. The primer pair was synthesized by Bangalore Genei Pvt Ltd. (Bangalore, India). The PCR was conducted in 25 µL volumes containing 1×PCR Buffer [100mM Tris–HCl, 100mM (NH4)2SO4, 100mM KCl, 1% TritonX-100,pH 8.8], 2.5mM Mg2+, 0.5 µM of each primer, 0.4mM dNTPs, 1 U Taq DNA polymerase (TaKaRa Bio., Kyoto, Japan), and 50 ng genomic DNA template. The amplification was performed in a Mastercycler nexus gradient (Eppendorf AG, Hamburg, Germany) with the following PCR program: 94◦C for 5 min, followed by 35 cycles of 94◦C for 45 s, 56◦C for 45 s, 72◦C for 1.5 min, and a final extension at 72◦C for 10 min. The PCR products were sequenced by NIRTH (Jabalpur, India).

Data Analysis

The original sequence was edited and assembled manually using CodonCode Aligner V3.0 (CodonCode Co., USA). The sequence was annotated and trimmed using ITS2 annotation tools based on the Hidden Markov Model (HMM) [30] to remove the conserved 5.8S and 28S DNA sequences [31]. BLASTA1 and the nearest distance method were used to evaluate the species authentication efficacy [32].

Results

Generation of Herbarium Sheet

For the morphological identification Herbarium voucher sheet has been prepared for the plant taken from the site. In herbarium identification the plant was identified as *Anisochilus carnosus* (Lf) which belongs to Lamiaceae family in the order lamiales.

Qualitative analysis of genomic DNA

To obtain good quality of DNA several modifications were done at every step and then final SOP was generated (Figure 1). As per as the optimized method is concerned we found very high yield around 123 µg per gram of leaf samples and the ratio at 260/280 nm found to be 1.82. Hence the yield as well as quality of the DNA was very good (Figure 2).

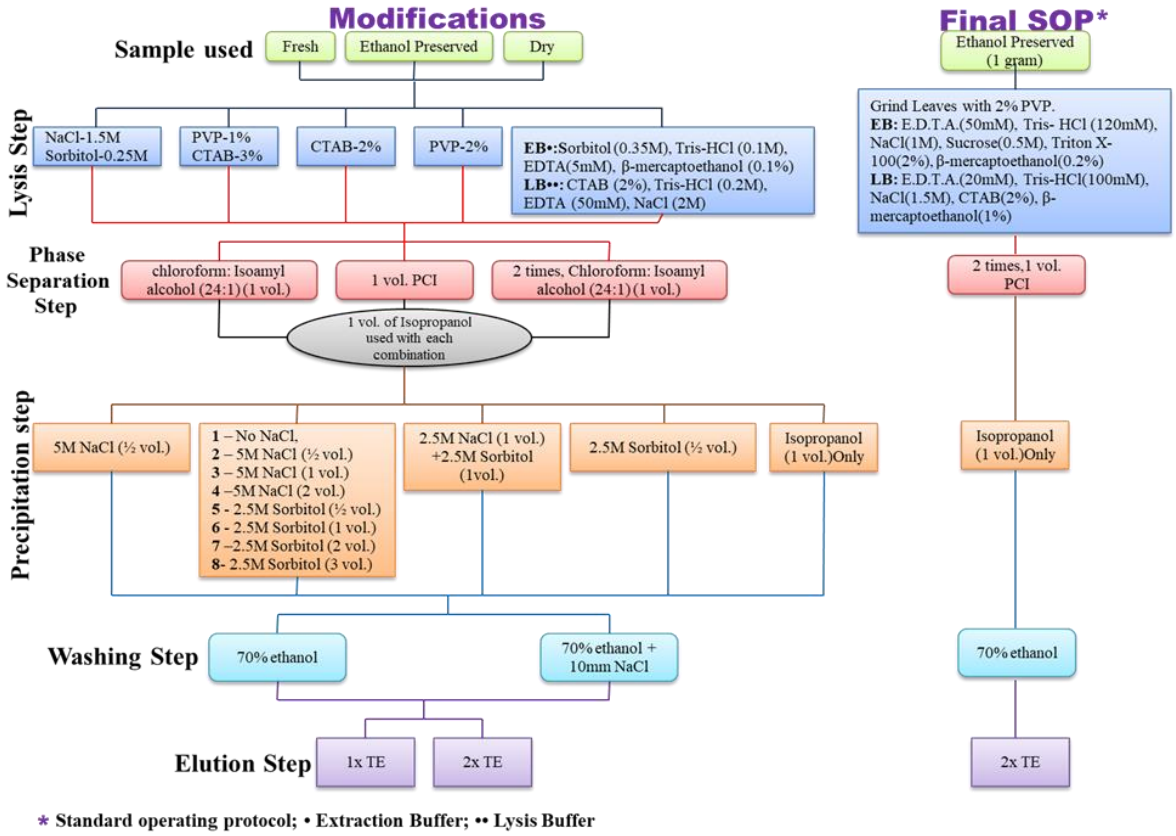

Fig 1: Diagrammatic representation of various modifications employed during the optimization for the isolation of genomic plant DNA from the leaf sample

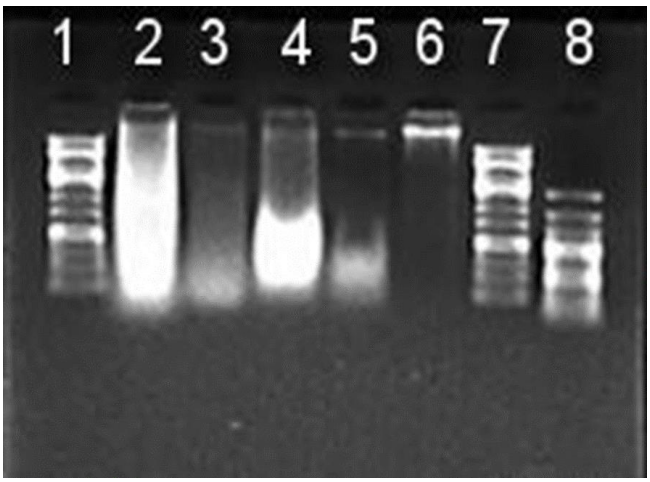

Fig 2: Gel electrophoresis of genomic DNA isolated form leaves of a plant on 1% agarose gel. Lane 1- 1kb DNA ladder. Lane 2, 3 & 4- Genomic DNA isolated by different combination. Lane 5- Genomic DNA isolated from present optimized method without RNase treated and Lane 6- Intact and bright DNA band isolated by optimized method treated with RNase.

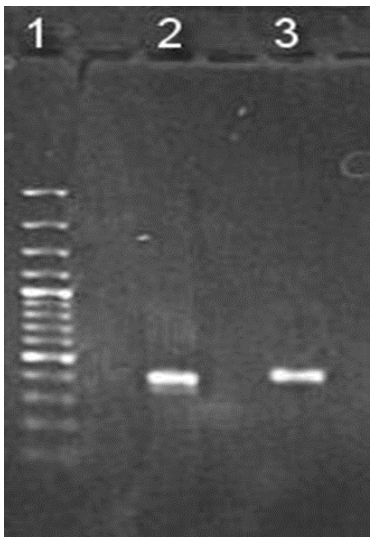

Fig 3: Amplification of DNA spanning 5.8s RNA-ITS2-28s RNA region. Lane 1- 100 bp DNA ladder. Lane 2- PCR product of ITS2F1 and ITS2R (390 bp); Lane 3- PCR product of ITS2F2 and ITS2R (418 bp).

Amplification and purification of ITS2 gene

To remove the RNA the isolated DNA was treated with RNase. To check the inhibitors in the sample DNA it was subjected to the restriction endonuclese digestion. There is no issue with the cutting of DNA with these enzymes which shows there are no enzyme inhibitors present in the sample DNA. Two PCR products of 419 and 390 bp were amplified by using ITS2F1, ITS2F2 as forward and ITS2R as reverse primers respectively. The size and intensity of the PCR amplified products were checked on to 2% agarose gel (Figure 3).

Sequencing and annotation of ITS2 region

A total of 20 ng of DNA was used as a template for generation the ITS2 gene sequences. Sequencing was performed along 5' as well as 3' end of the template for the authenticity of the sequence. The 419 bp sequences was annotated and trimmed using ITS2 annotation tool. Out of 419, 228 bp sequences were annotated as ITS2 for this plant. The structure for this ITS2 region was predicted. Further this annoted ITS2 sequence was subjected to BLASTA1 and the nearest distance method to construct phylogenetic tree (Figure 4).

Fig 4: Phylogenetic tree demonstrating differences in the ITS2 region of medicinal plants. The results are based on the maximum-likelihood approximation of the standard likelihood ratio test score. The branch length is proportional to the number of base pair substitutions. Scale bar indicates the base pair substitution rate.

Discussion

Genomic DNA was firstly extracted by the method described by Doyle and Doyle (1990). But the DNA was in very low quantity and very poor quality. The DNA bands were very faint and smearing. To obtain good quality of DNA so many modifications were done in Doyle and Doyle but smeared and very low intensity bands were obtained in the agarose gel. In this procedure we got sticky and mucilaginous brownish pellet due to high amount of polysaccharides and phenolic compounds get precipitated with DNA and due to which we encountered the problem of dissolving of pellet, it becomes viscous with TE buffer. There were so many combinations were also applied to precipitate high amount of DNA during modification but we got only less amount of DNA every time. To remove polysaccharides and phenolic compounds we used Sorbitol and PVP respectively in extraction buffer, but viscosity was not reduced. Different combinations of NaCl, Sorbitol with isopropanol were tried to precipitate the DNA. To obtain good quality of DNA several modifications were done at every step and then final SOP was generated. DNA quantity and quality was very good. The pellet obtained was clear and readily dissolved in 2X TE buffer. The DNA bands were intact and bright on agarose gel. The quantification of DNA was done by taking spectrophotometric readings at 260 and 280 nm. The non-optimized methods showed very less concentration of DNA ranging from 1 to 28 µg per gram of leaf samples. To check the purity 260nm/280nm ratio was measured. For non-optimized DNA samples, the ratio varies from 0.3 to 1.6 which shows contamination of the DNA. As per as the optimized method is concerned we found very high yield around 123 µg per gram of leaf samples and the ratio at 260/280 nm found to be 1.82. Hence the yield as well as quality of the DNA was very good. This method was further used to isolate DNA from other samples and get 58 to 110 µg DNA per gram of leaf samples. To remove the RNA the isolated DNA was treated with RNase. To check the inhibitors in the sample DNA it was subjected to the restriction endonuclese digestion. There is no issue with the cutting of DNA with these enzymes which shows there are no enzyme inhibitors present in the sample DNA. Two PCR products of 419 and 390 bp were amplified by using ITS2F1, ITS2F2 as forward and ITS2R as reverse primers respectively. The size and intensity of the PCR amplified products were checked on to 2% agarose gel. After confirmation of the size and yield of the PCR amplicons, it was further purified by using silica based column. After purification from agarose gel we got 3.6 µg of PCR amplified DNA. A total of 20 ng of DNA was used as a template for generation the ITS2 gene sequences. Sequencing was performed along 5' as well as 3' end of the template for the authenticity of the sequence. The 419 bp sequences was annotated and trimmed using ITS2 annotation tool. Out of 419, 228 bp sequences were annotated as ITS2 for this plant. The structure for this ITS2 region was predicted. Further this annoted ITS2 sequence was subjected to BLASTA1 and the nearest distance method to construct phylogenetic tree.

In herbarium identification the plant was identified as *Anisochilus carnosus* (Lf) which belongs to Lamiaceae family in the order lamiales. But in phylogenetic tree it grouped with plantaginaceae in the order lamiales. So at the first instance it looks the there is discrimination between the morphological and molecular characterization the species. Some studies have concluded that DNA barcoding sequences do not usually have sufficient phylogenetic signals to resolve evolutionary relationships [33]. So ITS2 region could be used as a DNA barcode but other regions should be investigated along with this to get the clear picture of identification of plants.

Conclusion

We would like to conclude that, we have developed an SOP for the isolation of genomic DNA from the carbohydrate rich leaf sample. Then the DNA barcode was developed for that plant by using the genomic DNA. By using the previously optimized methods we get smearing, less yield and no yield of DNA. Owing to this we did several modifications at every steps and get high-quality and excellent yield of DNA. By utilizing this DNA we develop the DNA barcode spanning ITS2 region and it was compared with other plants. In this it was showing close resemblance to the plants of plantaginaceae in the order lamiales. But In herbarium identification the plant was identified as *Anisochilus carnosus* (Lf) which belongs to Lamiaceae family in the order.

Acknowledgment

This work was supported by the St Aloysius College, Jabalpur (MP) India. So we are thankful to college for providing the space to carry out the work. We are also thanks to Mr. Mukesh Kumar for his technical help in this work.

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