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Original Article

Isolation of Genomic DNA From Six Species of *Phlogacanthus* Nees by Standardized CTAB Method

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Abstract

The *Phlogacanthus* Nees is an ethnomedicinal genus native to North-East India, rich in numerous phytochemical compounds that are pharmacologically significant. Plants from this genus have been used in traditional and herbal medicine. The CTAB extraction method has been employed for the preliminary molecular investigation, specifically for DNA isolation, as the first step towards advanced molecular studies. Total genomic DNA extraction and quantification were carried out in six species of *Phlogacanthus* Nees, namely *Phlogacanthus thyrsiflorus* Nees, *Phlogacanthus scurviflorus* (Wall.) Nees, *Phlogacanthus jenkinsii* C.B. Clarke, *Phlogacanthus quadrangularis* (Hook.) Heine, *Phlogacanthus guttatus* Nees, and *Phlogacanthus parviflorus* T. Anderson. The CTAB protocol, standardised for this genus, yielded a high quantity of DNA with better concentration and a purity ratio between 1.8 and 2.2, compared to the original standard protocol from fresh leaf tissue. The original protocol produced up to 220 ng/µl, while the kit protocol gave up to 600 ng/µl of DNA concentration. Therefore, the standardised CTAB protocol proved to be the most suitable method for rapid and efficient DNA extraction from the fresh leaf tissue of *Phlogacanthus* Nees, yielding between 100 and 1500 ng/µl of DNA—substantially higher than the other two protocols. In conclusion, the standardisation of the protocol results in high-quality, concentrated DNA, which is valuable for further molecular biological applications.

Keywords: *Phlogacanthus Nees, Ethnomedicinal, CTAB, Genomic DNA, Quantification*

Introduction

The genus *Phlogacanthus* Nees is a significant ethnobotanical resource in North-East India. Various ethnic communities in the region utilise members of this genus to treat a range of ailments. The leaves and roots of these plants possess several medicinal properties, including antipyretic, antidiabetic, anti-asthmatic, and analgesic effects. They are also used to prevent skin diseases such as soreness and scabies, and to cure colds, coughs, and jaundice (Phurailatpam *et al*., 2014). The pharmacological activities of the crude extracts from this genus are primarily attributed to the presence of chemical constituents such as phlogantholide and phlogacantholide. Other chemical compositions, including diterpene lactones, terpenoidal glycosides, and various phytochemicals, are also biochemically active compounds. As a result, *Phlogacanthus* Nees is in high demand, as many bioactive novel compounds remain to be discovered from the different species within the genus.

The genus *Phlogacanthus* Nees has been studied taxonomically, but little is known about the genes responsible for its phytochemicals and other molecular components such as RNA, proteins, enzymes,

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and other macromolecules. Therefore, the present study undertakes the standardisation of a method for DNA extraction from six species of *Phlogacanthus* Nees as the first step towards elucidating its molecular aspects. Despite its significant ethnomedicinal and pharmacological value, there is limited literature available regarding molecular studies on this genus. There are nine species and various germplasms of *Phlogacanthus* Nees found across different topographical areas of Assam, but only a few records exist.

Fresh leaves were chosen as the preferred plant material for DNA extraction using different protocols. Total genomic DNA extraction from the members of the Acanthaceae family, specifically from *Phlogacanthus* Nees, was carried out by modifying the original CTAB protocol and using leaves as the source material (McDade, Daniel & Kiel, 2008). However, as the plant species are highly rich in secondary metabolites, oils, and wax compounds, DNA extraction can be challenging, particularly during flowering seasons and wood growth. To overcome these difficulties, the CTAB protocol has been modified to improve results. In this study, three alternative and similar CTAB protocols were used to extract DNA from fresh leaf tissue of certain *Phlogacanthus* Nees species as the source material.

Material and Methods

Sample Collection

The plant species *Phlogacanthus thyrsiflorus* Nees, *Phlogacanthus curviflorus* (Wall.) Nees, *Phlogacanthus jenkinsii* C.B. Clarke, *Phlogacanthus quadrangularis* (Hook.) Heine, *Phlogacanthus guttatus* Nees, and *Phlogacanthus parviflorus* T. Anderson were collected from various locations in Assam, India, and were raised in the experimental garden of Handique Girls' College, Guwahati, Assam, India. The saplings were cultivated in earthen pots with moist garden soil, and fresh leaves were harvested for the experiment. *P. thyrsiflorus* Nees, *P. curviflorus* (Wall.) Nees, and *P. guttatus* Nees were collected from the Kamrup (Metro) district, while *P. jenkinsii* C.B. Clarke was obtained from Diphu Recreation Park in the Karbi Anglong district and other parts of the district. *P. quadrangularis* (Hook.) Heine was sourced from Nambor Forest, located on the border between the Golaghat and Karbi Anglong districts. *P. parviflorus* T. Anderson was collected from the Eastern Himalayan Botanic Ark at the Balipara Foundation, and *P. curviflorus* (Wall.) Nees was gathered from the Amsoi Forest Range and Silchang Forest Range. All plant species were authenticated by repositories at the Botanical Survey of India (BSI), Shillong, Meghalaya, India, and the Department of Botany, Gauhati University, Guwahati, Assam, India, by renowned taxonomists.

Reagents

Liquid nitrogen, isolation buffer (2X CTAB buffer), chloroform: isoamyl alcohol (24:1, v/v), isopropanol, TE buffer, 70% ethanol, proteinase K, and RNase solution were used in the experiment. The RNase solution was prepared following the standard method (Doyle & Doyle, 1987; Barman, Anshumali & Marak, 2017). First, 10 ml of RNase buffer was prepared by dissolving 10 mM Tris-HCl and 15 mM HCl in autoclaved double-distilled water (ddH_2O) to make the final volume. Then, the RNase solution (10 mg/ml) was prepared by adding 1 mg of RNase to 1 ml of RNase buffer and heating it in a water bath for 15 minutes to denature any contaminating DNase. The RNase solution was then allowed to cool slowly and was finally stored at -20°C.

Methodology

Fresh tender leaves were collected to perform the experiments and used as the starting material for all CTAB protocols. The first and second protocols were CTAB protocols involving the use of liquid nitrogen, while the third protocol employed the HiPurA® Plant DNA Isolation Kit (CTAB Method) by Himedia, without the use of liquid nitrogen. The protocol was slightly modified and standardised with adjustments to centrifugation and chemical composition. DNA quantification was carried out using a Nanodrop (Thermo Scientific 1C). All three methods were tested through a trial-and-error approach for all six species, with some changes in the procedure and chemical composition to minimise contamination in the DNA.

Results

Origin CTAB protocol (Doyle & Doyle, 1987; Shyu & Hu, 2013) – DNA was isolated from fresh leaves tissue following the method without any alteration in chemical ingredients and methodology steps as devised by Doyle and Doyle, 1987.

1. 500mg of leaves tissue was grinded and 400mg of grinded tissue in the liquid nitrogen was incubated in the pretreated 2XCTAB buffer (100mM Tris-HCl, 1.4M NaCl, 20mM EDTA, 2%CTAB, 2% w/v PVP40, and 0.2% β-mercaptoethanol) at 65°C for 1 hour.
2. Then, chloroform: isoamyl alcohol (24:1, v/v) was added to the mixer and blended.
3. Then, the mixture was centrifuged at 9,000rpm for 10 minutes and the aqueous layer was transferred to another centrifuge tube.
4. To this solution chilled isopropyl alcohol was added and incubated at -20°C for 30 minutes.
5. The DNA pellet was obtained by centrifuging the white precipitate at 9,000 rpm for 10 minutes.
6. Then, the pellet was washed in 75% ethanol and dissolved in TE buffer (0.5 EDTA and 1M Tris-HCl, pH 8.0).
7. RNase digestion was performed.

Standardised Protocol - This method is standardised from the above-mentioned original protocol after modifying in technical steps and composition of reagents for removing the contamination in the isolated DNA.

1. 2XCTAB buffer (200mM Tris-HCl, 1.4NaCl, 20mM EDTA, 2% w/v CTAB, 4%w/v PVP40 and 5% β-mercaptoethanol) was preheated at 60°C for 20minutes.
2. The plant material taken was 500mg of fresh leaves weigh and grinded in liquid nitrogen. Then from this ground tissue, 400g was weighed and transferred to a 2ml microcentrifuge tube. In the tube, 400µl of pre-warmed CTAB buffer is added and mixed by gentle inversion.
3. 5µl Proteinase K solution was added to the tissue and incubated at 60°C for 1 hour.
4. After the incubation the tissue material was allowed to cool down and 400ml of Chloroform: isoamyl alcohol was added to the tissue material and blended thoroughly.
5. Then, the material was centrifuged at 10,000 rpm for 10 minutes at 25°C.
6. After centrifugation the supernatant was transferred to a new 1.5 ml microcentrifuge tube and chilled isopropanol was added. It was incubated at -20°C for 30 minutes.
7. Then it was centrifuged at 4°C for 10,000 rpm for 10 minutes.
8. The pellet was obtained and washed in chilled 70% ethanol and dissolved in 100µl 1XTE buffer.
9. Then, RNase digestion was performed at 37°C for 30 minutes by adding 10µl RNase solution.
10. The gel was prepared with 0.8% agarose in 1X TAE buffer. Electrophoresis was done for 1 hour 30 minutes. The bands were observed in the Bio-rad gel documentation system with UV transilluminator.

DNA isolation from fresh leaf tissue by HiPurA® Plant DNA Isolation Kit (CTAB Method) by Himedia as per manufacturer's instructions without the use of liquid nitrogen (HiMedia Laboratories, 2023).

The three CTAB protocols, original CTAB protocol (Doyle & Doyle, 1987), standardised CTAB protocol (Doyle & Doyle, 1987), and HiPurA® Plant DNA Isolation Kit (CTAB Method) by Himedia were suitable for extracting total genomic DNA from the species of *Phlogacanthus* Nees. The original or standard CTAB protocol by (Doyle & Doyle, 1987) was standardised with the use of liquid nitrogen. In the two protocols, 500mg of fresh leaf tissue was weighed first and then 400 mg of ground tissue was transferred to CTAB buffer to yield a high amount of DNA that varied from species to species. 400 mg powdered ground material was taken for the reaction because some small amount of tissue became embrittled and shrunk during homogenisation or cryogenic grinding. The kit method, however, did not need liquid nitrogen but yielded a lower amount of DNA usually than the conventional method which needed very little amount of fresh leaf tissue. From the original protocol of Doyle and Doyle (1987) standardised protocol for DNA isolation mentioned here was modified for leaves material of different species of *Phlogacanthus* Nees suitable for PCR reactions. Fresh and tender leaves are essential

because they are free of any diseases and contain very few secondary metabolites (Ravindran, Gayan & Das, 2017).

The original protocol was standardised into extraction protocol with the requirement of plant material especially in the concentration of reagents of extraction buffer. Thus, the lysis of cells for the reaction was done using homogenised liquid nitrogen and then incubated in heat using lysis buffer in the two manual protocols. But in the kit method cell disruption and lysis were done in the suspension buffer. So, grinding in liquid nitrogen gave better quality and more concentration of DNA (Schenk *et al*., 2023).

The steps and composition of the CTAB buffer and reagents were modified from the original method. The concentration of PVP (Polyvinyl phosphate) had been increased from 2% to 4% to remove secondary metabolites and alkaloids. The concentration of Tris-HCl had been increased from 100mM to 200mM. The use of a high level of NaCl (1.4M) which is more than 0.5M in the extraction buffer in both the manual CTAB protocols was found to be efficient in removing polysaccharides (Sahu, Thangaraj & Kathiresan, 2012). The concentration of β-mercaptoethanol was increased from 0.2% to 5% to remove the polyphenols and tannins. This ensures the oxidation of phenols and the use of phenol with Chloroform: isoamyl alcohol was avoided as it may cause DNA degradation (Pooniya *et al*., 2019). Also, the use of chloroform is advantageous because it removes almost all organic compounds (Mavrodiev *et al*., 2021). It was found to be effective in denatured proteins in the tissue (Russo *et al*., 2022). Incubation in an isolation buffer was done at 60°C instead of 65°C. The centrifugation step after blending with chloroform isoamyl alcohol was done at 10,000 rpm at 25°C instead of 9,000 rpm. Second centrifugation which was required to be done after adding the isopropanol and incubation at -20°C for 30 minutes was done at 4°C for 10,000 rpm. The centrifugation time was increased from 9,000 rpm to 10,000 rpm because the DNA pellet was not obtained using the first speed i.e. 9000 rpm but the pellet appeared at the centrifugation speed of 10,000 rpm. The alcohol used for the washing step was 70% ethanol. The RNAase solution was prepared according to CTAB protocols (Doyle & Doyle, 1987; Barman, Anshumali & Marak, 2017), then, RNAase digestion was performed. The TE buffer used in the original method was prepared by mixing 0.5 EDTA and 1M Tris-HCl, pH 8.0 was also efficient but in the rest two methods 1XTE buffer was used.

The purity ratio of the DNA following the original CTAB (Doyle & Doyle, 1987) shows between 1.6-1.82 and yielded up to 220 ng/µl amount of DNA, proving the less efficiency of the protocol for isolation of DNA for further analysis. It showed a lower ratio which hints at the presence of contaminants such as phenols or proteins (Bailey *et al*., 2022). Also, DNA isolated with a standardised protocol showed a good purity ratio of the DNA ranging between 1.8-2.2 which showed that the CTAB protocol by Doyle and Doyle (1987) with modification is more efficient for obtaining the high amount of DNA from *Phlogacanthus* Nees than the other CTAB methods. The amount of DNA concentration yielded by the standardised CTAB method approximately ranged from 100-1500 ng/µl. On the contrary, the kit method showed a DNA purity ratio between 1.8-2.3 which shows that this method is also successful and suitable for this taxon but yielded less concentration of DNA than the standardised protocol between 100-600 ng/µl approximately. Moreover, in the kit and original method proteins were removed by only chloroform: isoamyl alcohol but the use of Proteinase K was found more effective. The standardised protocol shows good bands and yields a larger amount of DNA than the other CTAB methods with better purity results which earlier was difficult because of seasonal and physiological factors. This DNA isolation of other species of this genus is possible with the standardisation of this protocol. Table 1 shows the purity ratio and DNA concentration by the standardised protocol. Fig 1 shows the agarose gel electrophoresis of DNA samples of six species by the standardised CTAB protocol. Lane 1, 2, 3, 4, 5, and 6 are the bands for total genomic isolated DNA of the six species of *Phlogacanthus* Nees i.e. P. thrysiflorus, P. curviflorus, P. jenkinsii, P. quadrangularis, P. guttatus and P. parviflorus respectively. Therefore, a suitable time for isolating the DNA of the six species is between April to September because at this time white pellet is obtained as tender leaves are present. As, the remaining months are the reproductive phase of the plant species, so, during floral initiation and flowering, leaves are not suitable for DNA extraction because the plants are producing flowering pigments and plant hormones at peak during this period. So, many times during this phase sometimes colored pellet is obtained and also leaves are not

suitable for use as matured. The P. curviflorus gives pink colour pellets and P. jenkinsii and P. thrysiflorus give yellow-coloured pellets during this time. Therefore, due to seasonal factors, variation in DNA purity may occur.

1 2 3 4 5 6

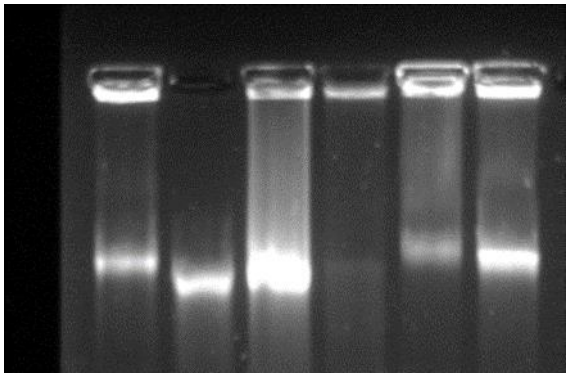

Figure 1: *Lanes 1, 2, 3, 4, 5, and 6 Loaded with DNA Samples Extracted from Six Species of Phlogacanthus Nees viz. P. thrysiflorus Nees, P. curviflorus (Wall.) Nees, P. jenkinsii C.B. Clarke, P. gutattus Nees and P. parviflorus T. Anderson by Standardised CTAB method (Doyle & Doyle, 1987).*

Table 1*: Table Showing Purity Ratio and DNA Concentration for six Species of Phlogacanthus Nees Done by Standardised CTAB Method (Doyle & Doyle, 1987).*

Taxon	Purity ratio	λ_{260}	λ_{280}	DNA Concentration ng/ µ l
Phlogacanthus thrysiflorus Nees	1.78	14.93	8.38	746.7
Phlogacanthus curviflorus (Wall.) Nees	1.79	17.23	9.64	861.7
Phlogacanthus jenkinsii C.B. Clarke	1.82	30.65	16.85	1532.5
Phlogacanthus quadragularis (Hook.) Heine	1.85	6.92	3.75	346
Phlogacanthus gutattus Nees	1.98	8.71	4.39	435.4
Phlogacanthus parviflorus T. Anderson	2.17	3.14	1.45	157.1

Discussion

The CTAB protocols are the most commonly used methods for DNA extraction from various types of plants and tissues. They are widely favoured due to their speed, efficiency, and ability to yield high-quality DNA from a variety of plant materials (Huang, Ge & Sun, 2000; Dawodu & Erinle, 2024). Fresh young leaf tissue is preferred for DNA isolation over other tissues because it contains fewer polyphenolic and terpenoid compounds than mature leaves and other tissues (Rosenthal & Janzen, 1979; Jobes, Hurley & Thien, 1995). In contrast, DNA extracted from mature leaves tends to be contaminated with high amounts of metabolites such as polysaccharides, tannins, and polyphenols, resulting in inferior quality DNA (Agarwal, Edhigalla & Walia, 2022). Different protocols use various methods to remove polysaccharides and polyphenols, which can vary depending on the taxa (Rogstad, 2003). The genus *Phlogacanthus* Nees is rich in various secondary metabolites, making it difficult to extract genomic DNA, which can reduce both the yield and quality of the isolated DNA, as phytochemical compounds tend to bind or co-precipitate with the DNA. During lysis, polyphenols may oxidise to quinones, leading to DNA degradation and fragmentation, which further precipitates with the DNA, thereby affecting its yield (Singh, Bhandari & Dwivedi, 2025). During the reproductive phase of the species, the production of pigment compounds peaks, causing precipitation with the DNA pellet and reducing the quality of DNA, making it unsuitable for extraction. Therefore, the ideal period for DNA

isolation is between April and July, when young leaves can be harvested, ensuring that a good amount of high-quality DNA can be obtained.

As reported previously, DNA isolation from foliage tissue often encounters impurities such as terpenes, polyphenolics, and polysaccharides, which are abundant in the tissue (Shepherd *et al*., 2002; Sinha & Singh, 2023). Consequently, perennial plants require more complex extraction methods compared to annual plants (Scott & Playford, 1996; Prasad, Ajinath & Mathew, 2022). The samples necessitate higher temperatures, such as 65°C, to promote tissue degradation and cell lysis, along with a prolonged water bath time of 60 minutes. An optimal precipitation step at -20°C is also essential for effective DNA isolation (Lui *et al*., 2025). The DNA extraction steps must be standardised for each species, as even minute variations in the position of DNA bands on the gel can occur due to differences in the genomic DNA size of the species. Therefore, isolating DNA using this protocol for a greater number of species within a given timeframe is a laborious task.

The CTAB methods generally yield a greater quantity of DNA compared to all other methods, including commercial kits (Shyu & Hu, 2013; Schenk *et al*., 2023). This is also true for *Phlogacanthus* Nees, as all species are perennial, with some being woody. Kit-based methods are gaining popularity due to their less labour-intensive nature, cost-effectiveness, suitability for small, less-equipped laboratories, and, most importantly, the reduced time required for the process (Agarwal, Edhigalla & Walia, 2022). However, the presence of polysaccharides and polyphenols in plants, which can vary between species, poses challenges during the DNA extraction process, as these compounds may interfere with isolation (Khanuja *et al*., 1999; Valiya Thodiyil, Edathumthazhe Kuni & Nediyaparambu Sukumara, 2024).

The CTAB-based DNA protocols are universally accepted because it uses reagents that are non-toxic and are environment friendly (Sheershika & Ram, 2024). The standardised CTAB DNA extraction protocol modified for the six species of *Phlogacanthus* Nees from earlier original CTAB protocol (Doyle & Doyle, 1987) is suitable for better results. The PCR analysis yielded high amount of DNA but, the kit method yielded was lesser in the amount of DNA possessing good quality. Therefore, the DNA extracted was found efficient for further downstream DNA analysis using various molecular markers such as RAPD, ITS, trnL-F etc. DNA extracted for analysis can be used in genetic diversity studies and germplasm identification of natural populations in medicinal plants which are unexplored and undiscovered. Conservation strategies to prevent genetic erosion and biodiversity loss is the need of the hour so, the present study aims at the basic molecular investigation first. It is suggested that this study would facilitate the scope for further improved approaches.

Conclusion

DNA isolation is the first step towards preliminary molecular investigation and the advanced biotechnological approach for improving plant species in various fields, such as DNA barcoding, DNA fingerprinting, genomics, and molecular cytogenetics. Thus, DNA studies provide crucial clues and information for research in areas like pharmacology, biotechnology, nanotechnology, gene editing, metagenomics, and epigenetics. With these considerations in mind, the present study on various taxa of *Phlogacanthus* Nees, which hold both medicinal and ecological importance, was undertaken. This research aims to provide a foundation for both in situ and ex situ conservation of their germplasm. Therefore, studies using PCR-based molecular markers and DNA fingerprinting will help in analysing plant germplasms rich in secondary metabolites, paving the way for advanced molecular research within the genus *Phlogacanthus* Nees.

Conflict of Interest

The authors declare that they have no competing interests.

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