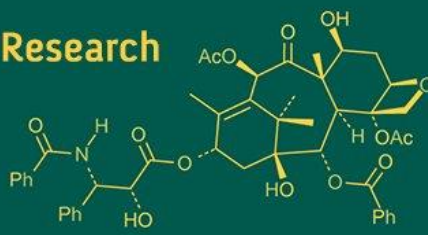


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**Shivakumar SN**  
Ph.D. Scholar, Department of  
Plantation, Spices, Medicinal  
and Aromatic Crops, College of  
Horticulture, Bengaluru,  
Karnataka, India

**K Hima Bindu**  
Former Principal Scientist,  
ICAR-Indian Institute of  
Horticultural Research,  
Bengaluru, Karnataka, India

**KV Ravishankar**  
Principal Scientist, Division of  
Biotechnology, ICAR-Indian  
Institute of Horticultural  
Research, Hessaraghatta,  
Bengaluru, Karnataka, India

**K Umesha**  
Former Dean, College of  
Horticulture, Bengaluru,  
Karnataka, India

**AP Mallikarjuna Gowda**  
Professor and Senior Farm  
Superintendent, Zonal  
Agricultural Research Station,  
University of Agricultural  
Sciences, GKVK, Bengaluru,  
Karnataka, India

**BN Maruthiprasad**  
Professor and Head,  
Department of Plantation,  
Spices, Medicinal and Aromatic  
Crops, College of Horticulture,  
Bengaluru, Karnataka, India

**Corresponding Author:**  
**Shivakumar SN**  
Ph.D. Scholar, Department of  
Plantation, Spices, Medicinal  
and Aromatic Crops, College of  
Horticulture, Bengaluru,  
Karnataka, India

## A modified CTAB method for isolation of high-quality genomic DNA from diverse genotypes of *Piper betle* L. for molecular analysis

**Shivakumar SN, K Hima Bindu, KV Ravishankar, K Umesha, AP Mallikarjuna Gowda and BN Maruthiprasad**

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### Abstract

*Piper betle* L. is an economically important perennial crop exhibiting considerable genetic diversity among cultivated genotypes. Reliable isolation of high-quality genomic DNA is an essential prerequisite for PCR-based molecular characterization. In the present study, a modified cetyltrimethylammonium bromide (CTAB) method was evaluated for genomic DNA isolation from 48 diverse genotypes of *Piper betle* L. Young leaf tissue was homogenized in CTAB extraction buffer supplemented with polyvinylpyrrolidone (PVP) and  $\beta$ -mercaptoethanol, followed by chloroform:isoamyl alcohol purification, isopropanol precipitation, ethanol washing and dissolution of the DNA pellet in TE buffer. The extracted DNA was evaluated for concentration and purity using a NanoDrop spectrophotometer. DNA concentration ranged from 1375.4 to 1802.3 ng/ $\mu$ L, with a mean of approximately 1610.4 ng/ $\mu$ L, while the A260/A280 ratio ranged from 1.77 to 1.87, indicating consistent DNA purity among the genotypes. The suitability of the extracted DNA for downstream molecular analysis was further demonstrated through SSR-PCR amplification, which produced clear amplification profiles across the evaluated genotypes. The modified CTAB protocol therefore provided a simple and reproducible approach for obtaining high-quality genomic DNA from diverse *P. betle* genotypes suitable for PCR-based molecular characterization.

**Keywords:** *Piper betle*, betelvine, genomic DNA, CTAB, DNA extraction, NanoDrop, SSR, PCR

### 1. Introduction

*Piper betle* L., commonly known as betelvine, is an important perennial crop belonging to the family Piperaceae. The crop is widely cultivated and has considerable economic, cultural and traditional importance. The cultivation of betelvine involves diverse locally adapted genotypes, which exhibit variation in morphological and other characteristics. Characterization and documentation of this diversity are important for effective utilization and conservation of betelvine genetic resources (Chattopadhyay and Maity, 1967; Parthasarathy *et al.*, 2009) <sup>[1]</sup>.

Molecular characterization provides a reliable approach for assessing genetic variation among plant genotypes. DNA marker technology has become an important tool for germplasm characterization and genetic fingerprinting. Previous molecular characterization studies in *P. betle* have employed RAPD and ISSR markers (Ranade *et al.*, 2002; Verma *et al.*, 2004; Patra *et al.*, 2011; Khadke *et al.*, 2015) <sup>[4, 6, 8, 9]</sup>. Among the different molecular marker systems, simple sequence repeat (SSR) markers are particularly useful for genetic diversity analysis and fingerprinting because of their co-dominant inheritance, multiallelic nature, high polymorphism and broad distribution across plant genomes. However, the success of PCR-based molecular marker analysis depends greatly on the quality of genomic DNA used as the template, as contaminants co-extracted during DNA isolation can inhibit PCR amplification (Demeke and Jenkins, 2010) <sup>[2]</sup>. The availability of DNA with adequate concentration, purity and integrity is therefore an essential prerequisite for reliable molecular analysis.

Isolation of genomic DNA from betleleaf tissues can be challenging because plant tissues contain polysaccharides, polyphenols and other secondary metabolites that may co-extract with DNA and interfere with its purification and subsequent molecular applications.

Phenolic compounds may undergo oxidation during tissue disruption and interact with nucleic acids, while polysaccharides and other contaminants can affect DNA quality and inhibit downstream enzymatic reactions such as PCR. These challenges can be particularly important when working with plant tissues rich in secondary metabolites, necessitating an extraction procedure capable of efficiently removing interfering compounds while maintaining adequate DNA yield and integrity (Doyle and Doyle, 1987; Porebski *et al.*, 1997) [3, 7].

The cetyltrimethylammonium bromide (CTAB) method is widely used for genomic DNA extraction from plant tissues. Modifications to the conventional CTAB procedure, including the incorporation of polyvinylpyrrolidone (PVP) and  $\beta$ -mercaptoethanol, can facilitate the removal of phenolic compounds and improve the quality of DNA obtained from plant tissues containing interfering secondary metabolites (Porebski *et al.*, 1997) [7]. In the present study, the CTAB procedure was modified by incorporating PVP during tissue homogenization and  $\beta$ -mercaptoethanol during extraction, followed by organic-solvent purification and alcohol precipitation. The modified procedure was used for isolation of genomic DNA from 48 diverse *P. betle* genotypes.

The extracted DNA was quantified using NanoDrop spectrophotometry by recording absorbance at 260 and 280 nm and calculating the A260/A280 ratio. The DNA was subsequently evaluated for its suitability for PCR-based molecular analysis using SSR markers. The objective of the present study was to evaluate the modified CTAB protocol for isolation of high-quality genomic DNA from diverse *P. betle* genotypes and to assess its suitability for downstream PCR-based molecular analysis.

## Objective

The objective of the present study was to evaluate a modified CTAB-based protocol for isolation of high-quality genomic DNA from diverse genotypes of *Piper betle* L. and to assess the suitability of the extracted DNA for PCR-based molecular analysis.

## 2. Materials and Methods

### 2.1 Plant material

Young and healthy leaves of 48 diverse genotypes of *Piper betle* L. were used for genomic DNA extraction. The genotypes represented diverse betelvine germplasm maintained for molecular characterization.

### 2.2 Chemicals and reagents

Molecular biology-grade chemicals and reagents were used for genomic DNA extraction. The major reagents included CTAB, Tris base, ethylenediaminetetraacetic acid (EDTA), sodium chloride,  $\beta$ -mercaptoethanol, polyvinylpyrrolidone (PVP), chloroform, isoamyl alcohol, isopropanol, ethanol and TE buffer. The CTAB-based extraction procedure incorporated PVP and  $\beta$ -mercaptoethanol to facilitate the removal of interfering compounds during DNA isolation.

### 2.3 Preparation of CTAB extraction buffer

CTAB extraction buffer was prepared using Tris-HCl (100mM), NaCl (1.5M), EDTA (20mM) and CTAB (2%).  $\beta$ -mercaptoethanol was added to the extraction mixture immediately before use. The buffer composition was

standardized prior to DNA extraction (Doyle and Doyle, 1987) [3].

### 2.4 Isolation of genomic DNA

Approximately 100 mg of young *P. betle* leaf tissue was finely ground in a chilled mortar and pestle. A small quantity of PVP was added during grinding to reduce interference from phenolic compounds. Subsequently, 500  $\mu$ L of pre-warmed CTAB buffer and 10  $\mu$ L of  $\beta$ -mercaptoethanol were added to the homogenate.

The homogenate was transferred to a 2.0 mL microcentrifuge tube and incubated at 60°C for 30 min, with gentle inversion after 15 min. Following incubation, the samples were centrifuged at 10,000 rpm for 10 min at room temperature, and the clear supernatant was transferred to a fresh microcentrifuge tube.

An equal volume (500  $\mu$ L) of chloroform:isoamyl alcohol (24:1) was added, and the contents were gently inverted until emulsified. The samples were centrifuged at 12,000 rpm for 5 min, and the upper aqueous phase was carefully transferred to a fresh tube. The chloroform:isoamyl alcohol extraction step was repeated to improve DNA purity.

An equal volume of ice-cold isopropanol (approximately 500  $\mu$ L) was added to precipitate the DNA, followed by incubation at -20°C for 2–24 h. DNA was pelleted by centrifugation at 12,000 rpm for 15 min. The supernatant was discarded, and the pellet was washed with 500  $\mu$ L of 70% ethanol. The samples were centrifuged at 12,000 rpm for 5 min, and the ethanol wash was repeated once.

The DNA pellet was air-dried to remove residual ethanol and subsequently dissolved in 50  $\mu$ L of TE buffer (pH 8.0). The extracted DNA was stored at 4°C or -20°C until further analysis (Doyle and Doyle, 1987; Porebski *et al.*, 1997) [3, 7].

#### 2.4.1 Preliminary evaluation of an alternative DNA extraction procedure

As part of preliminary method optimization, a silica dioxide-based procedure was initially evaluated for genomic DNA isolation from *P. betle* leaf tissue. DNA quality was assessed spectrophotometrically by recording absorbance at 260 and 280 nm. The obtained readings showed considerable variation in absorbance values and inconsistent A260/A280 ratios. Therefore, the silica dioxide-based procedure was not pursued further for downstream molecular analysis, and the modified CTAB protocol was subsequently selected for genomic DNA isolation and PCR-based validation.

### 2.5 Quantification and purity assessment of genomic DNA

The concentration and purity of genomic DNA were determined using a NanoDrop spectrophotometer. Absorbance was recorded at 260 and 280 nm, and the A260/A280 ratio was used as an indicator of DNA purity. DNA concentration was expressed as ng/ $\mu$ L. The DNA concentration and purity data obtained for the 48 genotypes are presented in Table 1.

### 2.6 Assessment of genomic DNA integrity

The integrity of the extracted genomic DNA was assessed by agarose gel electrophoresis. The DNA samples were separated on an agarose gel (0.8%) and visualized using a gel documentation system. The presence of distinct genomic

DNA bands with limited degradation was considered indicative of suitable DNA integrity.

## 2.7 PCR-based validation of extracted DNA

The suitability of the extracted genomic DNA for downstream molecular analysis was evaluated using SSR markers. PCR amplification was performed using genomic DNA as the template, and the resulting amplification products were resolved by agarose gel electrophoresis. A representative amplification profile obtained using the SSR marker Psol15 across the 48 *P. betle* genotypes is presented in Figure 1.

## 3. Results

### 3.1 DNA concentration and purity

The modified CTAB protocol successfully yielded genomic DNA from all 48 *P. betle* genotypes evaluated. The DNA concentration varied among the genotypes, ranging from 1375.4 to 1802.3 ng/μL. The lowest DNA concentration was recorded in CARI-2 (1375.4 ng/μL), whereas the highest concentration was recorded in IIHRBV68 (1802.3 ng/μL). The mean DNA concentration across the 48 genotypes was  $1610.4 \pm 114.2$  ng/μL. The A260/A280 ratio ranged from 1.77 to 1.87, with a mean value of 1.81, indicating relatively consistent DNA purity among the evaluated genotypes.

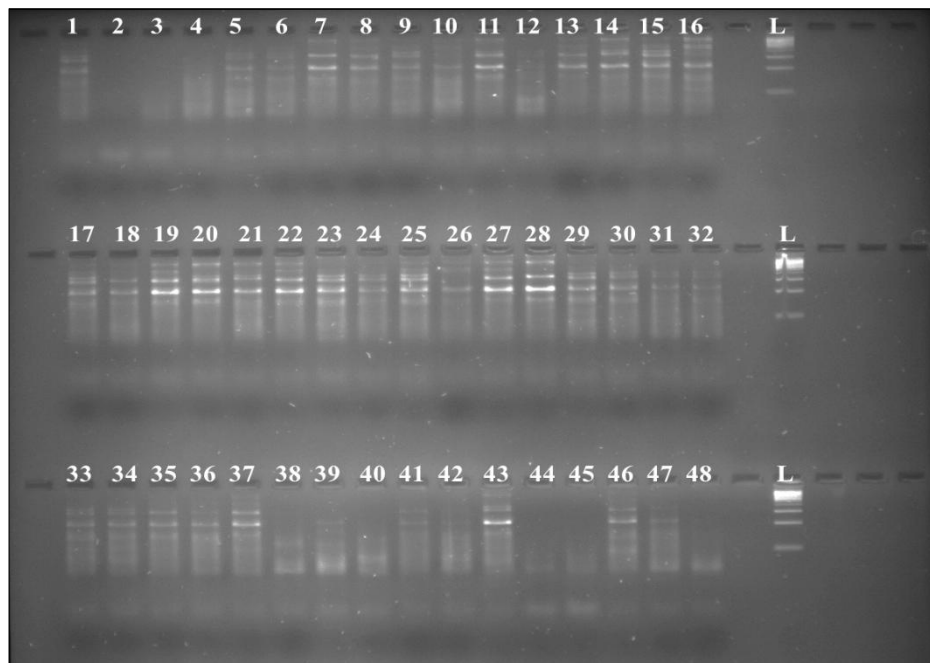
**Table 1:** DNA concentration and purity of 48 *Piper betle* L. genotypes isolated using the modified CTAB method

| Genotype            | A260 | A280 | A260/A280 | DNA concentration (ng/μL) |
|---------------------|------|------|-----------|---------------------------|
| BV-110              | 1.95 | 1.06 | 1.84      | 1542.3                    |
| BV-74               | 2.21 | 1.22 | 1.81      | 1745.9                    |
| BV-76               | 1.86 | 1.02 | 1.82      | 1460.7                    |
| BV-81               | 2.05 | 1.14 | 1.80      | 1624.5                    |
| BV-112              | 2.13 | 1.15 | 1.85      | 1720.1                    |
| IIHRBV67            | 1.89 | 1.01 | 1.87      | 1489.2                    |
| CARI-2              | 1.78 | 0.97 | 1.83      | 1375.4                    |
| IIHRBV68            | 2.25 | 1.26 | 1.79      | 1802.3                    |
| BV-75               | 2.00 | 1.10 | 1.82      | 1573.9                    |
| BV-96               | 2.10 | 1.19 | 1.77      | 1684.1                    |
| Bangla UP           | 1.97 | 1.10 | 1.79      | 1526.6                    |
| Karapaku            | 2.12 | 1.17 | 1.81      | 1713.4                    |
| Balipan             | 2.04 | 1.14 | 1.79      | 1598.0                    |
| Ramtekbangla        | 2.20 | 1.21 | 1.82      | 1760.2                    |
| Dhobabangla         | 2.16 | 1.19 | 1.81      | 1734.7                    |
| Desipan             | 1.90 | 1.06 | 1.79      | 1492.3                    |
| Nov Bangla          | 1.84 | 1.02 | 1.80      | 1420.9                    |
| Mohaba Bangla       | 1.99 | 1.09 | 1.83      | 1577.1                    |
| Calcutta Bangla     | 2.18 | 1.21 | 1.80      | 1782.0                    |
| Halisaharsanchi     | 1.92 | 1.06 | 1.81      | 1510.6                    |
| Bangla Nagaram      | 2.05 | 1.13 | 1.81      | 1654.4                    |
| Gujarath local      | 2.17 | 1.20 | 1.81      | 1751.6                    |
| Mysore local        | 1.91 | 1.03 | 1.85      | 1503.7                    |
| Sirugamani-1        | 2.09 | 1.16 | 1.80      | 1642.2                    |
| Simrali Babna Local | 2.00 | 1.08 | 1.85      | 1580.5                    |
| Simrali Babna       | 1.96 | 1.06 | 1.85      | 1548.0                    |
| Bangla ganmala      | 2.13 | 1.18 | 1.81      | 1721.3                    |
| Savanur local       | 1.87 | 1.02 | 1.83      | 1458.6                    |
| Bangla MP           | 2.22 | 1.23 | 1.80      | 1794.1                    |
| Khasipan            | 2.06 | 1.15 | 1.79      | 1659.8                    |
| Ghanegatte          | 1.98 | 1.10 | 1.80      | 1560.0                    |
| Meethapan           | 1.94 | 1.07 | 1.81      | 1530.7                    |
| Kali bangla         | 2.19 | 1.22 | 1.79      | 1772.9                    |
| Gachipan            | 1.88 | 1.03 | 1.83      | 1466.2                    |
| IIHR-PBH-7-24       | 2.01 | 1.10 | 1.83      | 1600.2                    |
| IIHR-PBH-7-28       | 2.11 | 1.17 | 1.80      | 1692.4                    |
| IIHR-PBH-7-25       | 1.93 | 1.06 | 1.82      | 1517.9                    |
| IIHR-PBH-6-1        | 2.14 | 1.19 | 1.80      | 1725.0                    |
| IIHR-PBH-7-37       | 2.00 | 1.09 | 1.83      | 1584.1                    |
| IIHR-PBH-7-36       | 1.95 | 1.06 | 1.84      | 1541.7                    |
| IIHR-PBH-7-10       | 2.16 | 1.20 | 1.80      | 1740.8                    |
| IIHR-PBH-6-11       | 1.89 | 1.04 | 1.82      | 1480.3                    |
| IIHR-PBH-9-12       | 2.07 | 1.14 | 1.82      | 1663.5                    |
| IIHR-PBH-9-13       | 1.86 | 1.02 | 1.82      | 1445.2                    |
| IIHR-PBH-9-16       | 2.15 | 1.19 | 1.81      | 1731.6                    |
| IIHR-PBH-8-52       | 2.03 | 1.13 | 1.80      | 1627.4                    |
| IIHR-PBH-6-4        | 1.91 | 1.05 | 1.82      | 1498.9                    |
| Ambadi Dabaspel     | 2.08 | 1.15 | 1.81      | 1672.6                    |

### 3.2 Integrity and suitability of genomic DNA for molecular analysis

The extracted genomic DNA showed detectable and relatively intact DNA profiles upon agarose gel electrophoresis. The DNA obtained using the modified CTAB procedure was subsequently used as a template for

SSR-based PCR amplification. The representative SSR profile shown in Figure 1 demonstrates amplification across the 48 *P. betle* genotypes. This provides practical evidence that the isolated genomic DNA was suitable for downstream PCR-based molecular analysis.



Lanes 1–48: individual *P. betle* genotypes; L: 100 bp DNA ladder.

**Fig 1:** Agarose gel electrophoresis showing amplification of SSR marker Psol15 across 48 *Piper betle* L. genotypes

#### 4. Discussion

The successful isolation of genomic DNA is a critical first step in PCR-based molecular characterization because the quality of template DNA directly influences the reliability of downstream amplification (Doyle and Doyle, 1987; Porebski *et al.*, 1997) [3, 7]. In the present study, the modified CTAB protocol yielded genomic DNA from all 48 *Piper betle* L. genotypes evaluated. DNA concentration ranged from 1375.4 to 1802.3 ng/μL, indicating substantial DNA recovery across the diverse genotypes. The variation in DNA concentration among genotypes may be associated with differences in tissue characteristics and genotype-dependent variation in the composition of cellular constituents. The highest DNA concentration was recorded in IIHRBV68 (1802.3 ng/μL), whereas CARI-2 (1375.4 ng/μL) recorded the lowest concentration.

The purity of the extracted DNA was assessed using the A260/A280 ratio. In the present study, the A260/A280 values ranged from 1.77 to 1.87, with most genotypes showing values close to 1.8. The relatively narrow range observed across the 48 genotypes indicates consistent DNA quality obtained using the modified extraction procedure. The relatively narrow range of A260/A280 ratios suggests consistent apparent DNA purity among the evaluated genotypes.

One of the important modifications incorporated into the extraction procedure was the use of PVP during tissue homogenization. PVP assists in reducing interference from phenolic compounds during plant DNA extraction (Porebski *et al.*, 1997) [7]. β-mercaptoethanol was also incorporated during extraction, followed by chloroform:isoamyl alcohol purification. The combined use of these components in the modified CTAB procedure likely contributed to the recovery of DNA with satisfactory purity suitable for downstream molecular analysis.

During preliminary optimization, a silica dioxide-based DNA extraction procedure was also evaluated for *P. betle*. However, the spectrophotometric assessment showed considerable variation in absorbance values and inconsistent

A260/A280 ratios across the tested samples. Based on these observations, the silica dioxide-based procedure was not continued for downstream molecular analysis. In contrast, the modified CTAB protocol consistently yielded genomic DNA with satisfactory concentration and A260/A280 ratios across the 48 genotypes.

The suitability of the extracted DNA was further demonstrated through SSR-based PCR amplification. Amplification products were successfully obtained from the extracted genomic DNA, indicating that the DNA was suitable as a template for PCR-based molecular analysis. The representative Psol15 amplification profile obtained across the 48 *P. betle* genotypes further demonstrated the applicability of the extracted DNA for SSR analysis. The presence of amplification products across the evaluated samples, together with the satisfactory spectrophotometric measurements, provides evidence that the modified CTAB procedure yielded DNA suitable for downstream molecular applications.

The combined assessment of DNA concentration, A260/A280 ratio and PCR amplification provides stronger evidence of DNA quality than concentration measurements alone. Although spectrophotometric measurements provide useful information regarding DNA quantity and apparent purity, successful amplification provides functional evidence that the extracted DNA is suitable for enzymatic reactions. Thus, the present study demonstrates that the modified CTAB procedure was effective not only in obtaining adequate quantities of genomic DNA but also in producing DNA suitable for PCR-based molecular analysis. Overall, the modified CTAB procedure provided a simple, reproducible and effective approach for genomic DNA isolation from diverse *P. betle* genotypes, yielding DNA of sufficient quantity and quality for PCR-based molecular analysis.

#### 5. Conclusion

The modified CTAB protocol evaluated in the present study successfully isolated genomic DNA from 48 diverse

genotypes of *Piper betle* L. The extracted DNA showed concentrations ranging from 1375.4 to 1802.3 ng/ $\mu$ L, with A260/A280 ratios ranging from 1.77 to 1.87. The incorporation of PVP and  $\beta$ -mercaptoethanol, together with chloroform:isoamyl alcohol purification and alcohol precipitation, yielded DNA of consistent quality across the evaluated genotypes. The suitability of the extracted DNA for downstream molecular applications was further confirmed by successful SSR-PCR amplification. Therefore, the modified CTAB protocol provides a simple, reproducible and effective approach for obtaining genomic DNA from diverse *P. betle* genotypes suitable for PCR-based molecular characterization.

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