Indian Journal of Entomology Online published Ref. No. e24155 DoI. No.: 10.55446/IJE.2024.2155

DNA ISOLATION FROM SIX SPOTTED LADYBIRD BEETLE *CHEILOMENES SEXMACULATA* (F)

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ABSTRACT

DNA extraction is a routine step in many insect molecular studies and extraction methods need to be evaluated for their efficiency, cost, and side effects, such as DNA degradation. Individuals of six spotted ladybird beetle, *Cheilomenes sexmaculata (F.)* were subjected to DNA extraction by the CTAB method and PureLink® Kit. The methods were compared in terms of DNA quantity and quality, cost of materials, and time consumed. The CTAB method resulted in higher DNA yield (ng DNA) at a much lower price and less degradation, as revealed on agarose gels. The PureLink® Kit was the most time-efficient but costliest, and degradation was observed on agarose gels. The DNA samples obtained were tested on agarose gel PCR for six SSRs located in various positions of the beetle's genome. The results revealed that DNA isolated from the CTAB method showed successful amplifications, but the PureLink® Kit method did not show any amplification. These evaluations guide the choice of DNA extraction methods from *C. sexmaculata* beetles.

Key words: *Cheilomenes sexmaculata*, coccinellid beetles, CTAB method, diversity, DNA Extraction, DNA sequencing, PureLink® kit

DNA extraction is a routine step in many biological studies, including molecular identification, phylogenetic inference, genetics, and genomics. Therefore, various methods have been standardized to isolate total DNA from biological materials. An ideal extraction technique should optimize DNA yield, minimize DNA degradation during extraction, and be efficient in terms of cost, time, labor, and supplies. It must also be suitable for extracting multiple samples and generating minimal hazardous waste. The cetyltrimethylammonium bromide (CTAB) and PureLink® methods are widely employed for DNA extraction from various organisms, including insects (Milligan and Hoelzel, 1998). In contrast, the PureLink® Kit employs two solutions, salt and detergents (Obinata et al., 2014). The PureLink® kit employs a spin-column with a DNA-binding membrane and a buffer system for cell lysis, DNA binding, and elution (Hancock et al., 2008). Typically, sodium ions are used to precipitate DNA from its aqueous solution, commonly employing absolute ethanol or isopropanol (Waldschmidt et al., 1997; Chen et al., 2008). *Cheilomenes sexmaculata* (F.) (Coleoptera: Coccinellidae), is common among all ladybird beetles well known for predating on sucking pests in general and aphids in particular. Extraction of DNA is involved in a variety of applications related to the beetle's genetics and molecular toxicology, which helps in selecting the better strains for classical and augmentative biological control (Miller et al., 2009). The quantity and quality of DNA isolated from individual beetles varied considerably among extraction methods. Therefore, a comparison of both PureLink® Kit and CTAB methods was conducted to optimize DNA extraction. Also, investigated the effects of ethanol volume, temperature, and incubation time on DNA precipitation. The factors affecting DNA yield and quality were stressed.

MATERIALS AND METHODS

The present study was conducted in the Division of Entomology, ICAR- IARI, New Delhi. The *C. sexmaculata* were collected from five zones (North, South, East, West, and Central) of India. For each zone, five sub localities were selected *viz.,* North (Delhi, Haryana, Jaipur, Jhansi, and Karnal), Central (Nagpur, Akola, Amravati, Wardha, and Chandrapur), East (Cooch Behar, Jorhat, Barrackpore, Lembucherra, and Barapani) West (Navsari, Dandi, Anand, Junagadh, and Vapi) and South (Coimbatore, Bengaluru, Coorg, Tiptur, and Mudigere) from June 2018 to December 2019. The

collection includes both male and female individuals. Later, the samples were stored in 70% alcohol and frozen at -20°C for further molecular analysis (Kim *et al*., 2012). For each method, total DNA was extracted from ten beetles, including five females and five males. The colour of the DNA pellet in each tube was recorded. The DNA from single beetle was re-suspended in 100 ml of molecular-grade water. The DNA extraction buffer (DEB) contained 0.5 M EDTA (pH 8.0), 1M Tris-HCl (pH 8.0), 4 M NaCl and 10% CTAB, mixed in the proportion of 20:50:175:100 ml and made to a volume of 500 ml using double distilled water. The powdered samples were taken in 2 ml micro-centrifuge tubes containing 1.5 ml of pre-warmed (65°C) DEB, 2.0µl of 2-beta-mercaptoethanol, and 10 ul of 20% SDS. Subsequently, 3 ul of proteinase-K was added. The tubes were placed in Thermo shaker at 65°C for overnight digestion with shaking speed at 250 rpm. After incubation, centrifuge at 14,000 rpm for 10 min. The supernatant was taken along with an equal volume of Phenol: chloroform: Isoamyl alcohol (25:24:1). After that, transferred the clear upper solution in a new micro centrifuge tube in addition to this, added matching volume (~1 ml) of chloroform: isoamyl alcohol mix (24:1) and centrifuged for 10 min at 4°C with 10,000 rpm. The clear supernatant was collected, to which $1/3^{rd}$ of ice-cold isopropanol was added and incubated at -20°C for 2 hr. The pellet was washed twice with 70% ethanol. Then the pellet was dried at room temperature for 15 min at 30°C, dissolved in TE buffer (100 mM Tris and 50 mM EDTA, pH 8.0) sufficiently depending on the DNA pellet size, and stored at 4°C after complete dissolution (Zeugin and Hartley, 1985).

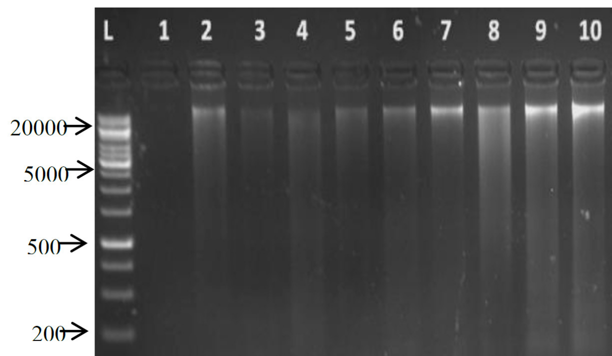
The PureLink® Kit contains two primary reagents: cell lysis and protein precipitation solutions (Thermo Fisher Scientific Systems, Massachusetts, USA). For each beetle, 500 ml of the lysis solution and 5 ml of proteinase-K solution (20 mg/ml) were used. After homogenization, the lysate was incubated at 65°C for 20 min. The procedures of cell lysis, RNase A treatment and protein precipitation followed the manufacturer's protocol with necessary modifications according to the beetle weight range (Invitro systems, 2004). DNA precipitation and drying were done as in the CTAB method. A NanoDrop® ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) was used to measure the DNA concentration and the absorbance ratio was A260/A280. A pure DNA sample has a ratio of 1.8 and is relatively free from protein contamination (Crouse and Amorose, 1987). To compare the efficiency of the DNA extraction methods, the DNA yield from single beetle was calculated based on the DNA concentration and final volume. To visualize DNA quality, 250 ng of each DNA was loaded on a 0.5% agarose gel at 45 volts for 2 hr. The extracted DNA sizes were estimated using the DNA marker of GeneRulerTM 1 kb and 50 bp (PCR amplifications) (Fermentas, Glen Burnie, MD, USA). A digital image was taken under UV light in a Universal Hood II (Bio-Rad, Hercules, CA, USA). To assess the DNA quality for PCR application, a set of six SSRs markers (CsM43755, CsM29435, CsM45955, CsM81952, CsM48497, and CsM30177) were amplified from each DNA sample following the protocol as suggested by Kim et al., (2008). The cost analysis for each method was based on the prices of chemicals, enzymes and disposable items required for a single beetle DNA extraction. Extraction times for both methods were recorded, excluding solution preparation time in the CTAB method (Rohland et al., 2004). The CTAB method involved a separate group of beetles, with 600 ml CTAB solution used per extraction, and the resulting supernatant was divided into 15 tubes, each containing 30 ml. These tubes were subjected to various treatments, including testing three different ethanol-to-lysate volume ratios. Following ethanol addition, the tubes were inverted 10 times, and DNA pellet precipitation involved centrifugation at 12,000 g for 15 min, followed by washing and drying as per the CTAB method. The DNA pellets were subsequently resuspended in 30 ml molecular-grade water for further analysis. The effects of extraction methods on DNA yield rate, absorbance ratio, DNA yield under different precipitation conditions and the impact of ethanol volume and temperature were evaluated using the XLSTAT version 2021.5 extension in Excel 2007.

RESULTS AND DISCUSSION

The CTAB method recorded a relatively higher DNA yield rate (ng/mg) (mean ±SE, 1800±27), as compared to the PureLink® Kit method (600±12). The mean absorbance ratio was highest for CTAB method (>1.8), while the mean ratio of the PureLink® kit was the lowest, indicating the highest protein contamination. In CTAB methods buffers prepared in the laboratory were used which resulted in higher DNA yield rates and less degradation. The DNA extracted by the PureLink® Kit contained the highest protein contamination, as indicated by the absorbance ratio. With the shortest time spent for a single extraction, the PureLink® Kit was the most convenient. In general, PureLink® Kit did not generate hazardous waste containing phenol and chloroform and did not require a fume hood to

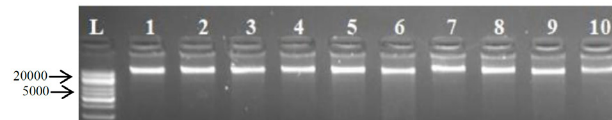
operate. For animal (insect) tissue, typical yield rates ranged from 1000- 5000 ng/mg (Gilbert et al., 2007). DNA yield is subject to numerous factors, including species, tissue type, preservation methods, extraction procedures, and precipitation techniques. In the case of tobacco budworm, *Heliothis virescens* abdomens using the CTAB method, the yield rate falls within the range of 500-600 ng/ mg (Shahjahan et al., 1995). Modified CTAB extraction from integument tissue of the sea buckthorn carpenter moth (*Holcocerrus hippophaecolus*) yielded 2000-3000 ng/mg (Chen et al., 2008). In our study with *C. sexmaculata* beetles, the CTAB method yielded 1800 ng/mg, while the PureLink® Kit resulted in a lower yield of 600 ng/ mg. The temperature during lysate incubation can significantly affect DNA quantity and quality. Shahjahan et al. (1995) found that incubating at 37°C produced over double the total DNA compared to other temperatures, with the lowest mean absorbance ratio at 1.72. However, higher temperatures during lysis can lead to DNA degradation (Steiner et al., 1995). It's worth noting that the effect of incubation at 65°C should be confirmed for different organisms, as the CTAB method commonly employs temperatures ranging from 45 to 65°C (Linton et al., 2001).

The colour of precipitated DNA pellets differed within each DNA extraction method but varied more widely between the methods. The pellet colours range from clear, white, and yellow (CTAB method) to brown (PureLink® Kit). The brown colour of DNA pellets indicated the presence of protein contamination. Typical examples of DNA extraction using PureLink® Kit and CTAB methods visualized on a 0.5% agarose gel. The prominent bands of DNA were around 45 kb in size. The CTAB method showed significantly lighter smear tails, indicating no DNA degradation, and distinct bands appeared (Fig. 1). Still, the PureLink® kit showed darker and heavier smear tails indicating high DNA degradation, and cannot be used in library preparation while sequencing (Fig. 2). The colours of DNA pellets obtained at the end of extractions did not indicate the levels of protein contamination. The colors varied among extraction methods, probably due to the status of biological materials used for DNA extraction (Chomczynski et al., 1998). The set of six SSRs, 250-400 bp, were all successfully amplified from the 10 DNA samples extracted by the CTAB method (Fig. 3a). Still, unfortunately, we did not notice any amplification in the PureLink® Kit method (Fig. 3b), indicating that the isolated DNA was not of sufficient quality for PCR application. Regarding the DNA quality for molecular application, PureLink® Kit did not provide enough DNA for PCR, as demonstrated by six SSRs amplifications in this study. With an estimated size of 2.5 Gb, the beetle's genome has been proposed to be sequenced using the novel parallel sequencing technology Illumina® (Miller et al., 2010). To prepare the DNA library, extracted genomic DNA needs to be fractioned into smaller fragments (27- 42 bp for Illumina®) (Clark *et al.,* 2001). Therefore, the higher levels of DNA degradation during the extractions should not affect the DNA application in the Illumina®. In general, an extraction method should be tested for the follow-up molecular application before a large-scale extraction of DNA.



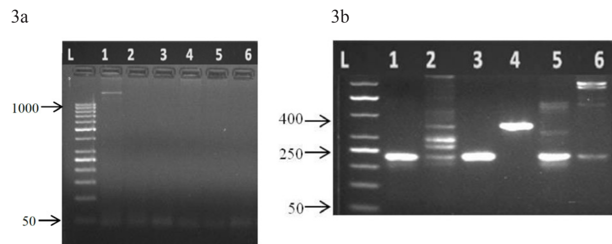
GeneRuler™1 kb DNA Ladder (L, in bp) and *C. sexmaculata* DNA samples from 1-5 (Females) and 6-10 (Males) isolated by PureLink® Kit (1-10).

Fig. 1. DNA electrophoresis on 0.5% agarose gel at 45 volts for 2 hrs in PureLink® Kit



GeneRuler™1 kb DNA Ladder (L, in bp) and *C. sexmaculata* DNA samples from 1-5 (Females) and 6-10 (Males) isolated by CTAB method (1- 10).

Fig. 2. DNA electrophoresis on 0.5% agarose gel at 45 volts for 2 hrs in CTAB method



GeneRuler™ 50bp DNA Ladder (L, in bp) and *C. sexmaculata* DNA sample against six SSRs markers (1-,2-).

Fig. 3. PCR amplifications on 1.5% agarose gel at 90 volts for 45 min in PureLink® Kit (3a) and CTAB method (3b).

Cost and time estimates for extracting DNA from a single beetle using both methods in Indian Rupees (INR) and hours (hrs) revealed that the laboratory-prepared CTAB method is notably cost-effective, ranging from approximately 52.36 to 65.44 INR/ sample, while the PureLink® Kit is comparatively more expensive at 202.50 to 219.76 INR. However, the PureLink® Kit requires less extraction time (1.3 hr) compared to the CTAB method (3.2 hr). When calculating the cost of DNA extraction for a specific number of samples using either method, you can multiply the cost per sample by the number of samples. However, for an accurate time estimate, one should consider the incubation and centrifugation times required to process all the samples. It's important to note that the time for buffer preparation in both the PureLink® Kit and CTAB methods was not included in this study, but it should be taken into account, especially when extracting DNA from only a few samples (Aljanabi et al., 1997).

Incubation periods impacted DNA precipitation, while ethanol volume and temperature directly affected DNA yield. Significant variations were observed among different ethanol volumes (60 vs. 80 vs. 100ul) and temperatures (4 vs. -20 vs. -80°C) used for precipitation. Among the conditions tested, the highest DNA yield (1850 ng/ mg) was achieved with 80ul of chilled ethanol at -20°C. The main effects plot highlights that the optimal conditions for DNA precipitation in this study were 80ul ethanol and -20°C. Larger ethanol volumes (80ul) and lower temperatures (-20°C) improved DNA yield but may not be suitable for lower DNA concentrations as they can slow down aggregation during centrifugation and increase costs. Generally, volumes of 60-80ul ethanol are recommended. Incubation time significantly influenced precipitation, with overnight incubation recommended for small DNA amounts (15 mg) (Miller et al., 2009). Raising the temperature from -20°C to -80°C notably enhances recovery (Phillips et al., 1995). DNA yield and quality are heavily reliant on the starting material's condition (fresh or old samples). For preservation, storing at -20°C in 70% ethanol for 6-8 months is advisable (30). The CTAB method, used in this study, is suitable for DNA extraction from various preserved specimens, including alcohol-preserved and air-dried museum samples. CTAB method to extract DNA from 6-month-old *C. sexmaculata* was employed (Gilbert et al., 2007). Yields from preserved specimens ranged from 3-5 mg/beetle, showing no degradation (indicated by no smears of 100-200 bp on agarose gels). In contrast, the PureLink® Kit exhibited significant degradation on agarose gels and no amplification with SSR markers. This study demonstrates that the CTAB method excels in terms of quality, quantity, cost, and time when extracting DNA from *C. sexmaculata*, making it the preferred choice for sequencing and molecular studies.

ACKNOWLEDGEMENTS

The first author acknowledges the Senior Research Fellowship received from the RGNF-NFST, MoTA, New Delhi and Authors also acknowledge facilities received from the Division of Entomology, ICAR-IARI, New Delhi, Govt. of India.

FINANCIAL SUPPORT

This research received no external funding.

AUTHOR CONTRIBUTION STATEMENT

SSS designed the experiments; HSR performed the experiment, to which DR contributed; HSR wrote the manuscript; SSS, SC and KMC helped in reviewing the manuscript.

CONFLICT OF INTEREST

No conflict of interest.

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(Manuscript Received: April, 2024; Revised: April, 2024;
Accepted: April, 2024; Online Published: April, 2024)
Online First in www.entosocindia.org and indianentomology.org Ref. No. e24155