Bulletin of Pure and Applied Sciences
Vol.39A (Zoology), No.1,
January-June 2020: P.88-93
Original Research Article

Print version ISSN 0970 0765
Online version ISSN 2320 3188
DOI 10.5958/2320-3188.2020.00011.X
Available online at www.bpasjournals.com

An Efficient Method of Genomic DNA Extraction and Quantification from Insects

[1]Dipti Kashyap*
[2]Kamal Jaiswal

Author's Affiliation:
[1,2]Department of Zoology
(Parasitology and Sericulture Laboratory)
Babasaheb Bhimrao Ambedkar University
(A Central University),
Lucknow, U.P (India)

***Corresponding author:**
Dipti Kashyap
Department of Zoology
(Parasitology and Sericulture Laboratory)
Babasaheb Bhimrao Ambedkar University
(A Central University),
Lucknow, U.P (India)

E-mail: hdgen31@gmail.com

Received on 24.01.2020
Accepted on 07.04.2020

Abstract:
The DNA extraction process is the most crucial step for demonstrating various molecular techniques such as PCR, DNA barcoding, metagenomics etc. This paper describes a simple and efficient protocol of DNA extraction from insect's sample that included Honey bees, Carpenter bees and Flour beetle's tissues with slight modification in CTAB method. DNA concentration together with purity has been determined by spectrophotometer absorbance reading at O.D260nm and O.D260/280nm respectively. Additionally, lambda DNA marker (NEB3012S) has been employed to validate band intensity by agarose gel electrophoresis. The DNA band exposed less smearing and intense band in agarose gel. Band of DNA in HB and CB lane was greater than 95.4ng but less than 95.4ng in lane FB as compared to lambda DNA marker bands. Also, DNA extracted from the tissue of Honey bee (HB), Carpenter bee (CB) and Flour Beetle (FB) measured by spectrophotometer for analysing O.D at 260 nm was found to be 4.9, 5.1 and 3.7ug/ml and the purity by considering 260/280 O.D ratio was 2.33, 1.76, 1.92. Absorbance ratio of 2.33 in DNA of Honey bees (HB) sample indicated slight contamination of protein or carbohydrate. Therefore, an achievable approach has been made to extract high quality of DNA by CTAB method and applying spectrophotometer for Optical Density as well as marker lambda DNA by agarose gel electrophoresis for quantity and quality analysis.

Keywords: Agarose gel electrophoresis, insects, optical density, quantity, quality

INTRODUCTION

Insects are vital constituent of the ecosystem. These are the major pollinators of agriculture, pests of plants and vectors of many diseases. To understand these aspects of insect biology and behaviour, insect identification is the elementary step and molecular entomology study is obligatory. Several protocols of DNA extraction methods have been developed for isolating DNA from plants and animal tissues however, in contrast to plant and animals, molecular entomologic studies lack optimized protocol of DNA extraction. Therefore, for molecular entomology studies and for establishing genetic and adaptive diversity within natural population of insects, it is required to develop a standardized genomic DNA extraction protocol. DNA extraction is a crucial step in generating DNA barcodes of insects (Ball and Armstrong, 2008) but the presence of hard chitinous exoskeleton and poly-phenolic

derivatives, the extraction of DNA from insect's tissue gets intricate (Arakane et al., 2005).In the present study, DNA from insect tissues of the order hymenoptera and coleopterans were extracted from a modified universal CTAB method and analysed for concentration and purity on agarose gel as well as by UV spectrophotometer. In the first part of the experiment DNA extracted has been assessed by using lambda DNA marker on agarose gel. In the second part, O.D measurement at wavelengths 260, 280 nm and 260/280 ratio has been considered for determining quality and quantity of DNA. For identification and characterization of insects, PCR based molecular technique has become the method of choice so high quality genomic DNA is required for analysing PCR based applications (Singh et al., 1998) and CTAB method has been used widely to extract DNA from plants (Doyle and Doyle, 1990) and in the present research a slight modification in CTAB method has been demonstrated to extract DNA from insect's tissue.

MATERIALS AND METHODS

Insect homogenate preparation:
The insects were collected and preserved at -20°C deep freezer (REMI.RQV-200 PLUS). Before preparing insects homogenate, the collected and preserved insects were washed with 70% ethanol stored at 4ºC properly, then immediately crushed using PBS by mortar and pestle individually. The entire crude homogenate filtered through muslin cloth and the filtrate was centrifuged (REMI-R-24 centrifuge) at 3000rpm for 15 minutes (Kyei-Poku et al., 2008). The pellet was collected and supernatant discarded. 1ml of ddH_20 was added to the pellet, vortex and centrifuged. The process was repeated several times to get a clear homogenate.

Preparation of Cell Lysis Buffer:
100mM Tris-HCl, pH=8; 2MNaCl; 20mM EDTA; 2.5% w/v CTAB; 3% Beta mercaptoethanol in 100mL of distilled water

DNA Extraction:
Extraction of DNA was carried out with slight modifications by CTAB method (Doyle and Doyle, 1987). 1000µl of lysis buffer was added to the homogenate (pellet) in 2ml eppendorf tube. Vortex and kept aside for five minutes. 50µl of Proteinase K and 25µl of RNase were added to the mixture and heated up to 55-65°C for 3-4 hrs and was left overnight. After lysis buffer treatment the homogenate was vortex and centrifuged for 1min at a speed of 14000 rpm. The supernatant was transferred to fresh 2ml tube (1ml in each tube). Equal volume of Phenol, Chloroform and Isoamyl-alcohol (P:C: I) in ratio of 25:24:1was added in the supernatant. After adding P:C:I, the tubes were inverted several times and centrifuged at 14000 rpm for 15 mins. Two layers formed and upper layer was transferred to 2ml fresh tubes. 1ml ethanol and 50µl of 3MSodium acetate (NaOAc) was added and vortex to mix. Further, incubated at 4°C for 30mins and again centrifuged at 14000 rpm for 30mins. DNA pellet settled and the supernatant was carefully decanted off. DNA pellet was washed with 1ml of 70% ethanol. Centrifuged at 14000 rpm for 3 mins.The supernatant was decanted. DNA pellet was let for air drying and once dried, 50µl of autoclaved ddH_2O was added and mixed by thawing. For prolonged usage it was preserved at -20°C. The overall extraction procedure has been performed at room temperature.

Agarose Gel Electrophoresis:
0.8% of agarose gel was prepared [0.8g agarose powder (BR Biochem) dissolved in 100ml of 1XTAE buffer] and heated on hot plate, allowed to cool around 60°C temperature, 5µl of EtBr (10mg dissolved in 1000µl sterile distilled water) was added and mixed properly and poured in casting tray with comb in place. The 0.8% gel when solidified, was transferred to electrophoresis unit filled with 1XTAE buffer and 5µl of DNA with 1µl of 6X DNA loading dye (Bromophenol blue; Xylene Cyanol, Thermofisher Scientific) and were run at a voltage of 60V. The DNA band was observed under UV trans-illuminator (GeNei TM) at 254nm.

DNA Quantification:
Quantification of extracted DNA was done by spectrophotometer (Biophotometer D-30) and samples were also checked on 0.8% agarose gel by lambda DNA marker (NEBN3012S Digested Hind3).

Protocol for Spectrophotometer reading:

- 1µl of DNA was diluted in 99µl of ddH_2O
- ddH_2O was used as control for calibration
- Readings were taken at 260nm and 280nm
- 1O.D at 260nm for dsDNA=50ng/µl
- Concentration was checked by the formula: $O.D_{260}$×50ng/µl× dilution factor
- Purity of DNA was checked by $O.D_{260}/O.D_{280}$ ratio that has value between1.8-2.

RESULTS

In the present work, we isolated genomic DNA from insect's tissue (Figure 1) from Honey bee (HB), Flour Beetles (FB) and Carpenter bee (CB) by CTAB method that was modified from Doyle and Doyle (1987) protocol of DNA extraction. Intense DNA band with slight smearing was observed in agarose gel image in the lane HB and CB. Less intensity band was observed in FB lane. Additionally, with Lambda DNA marker, all three samples had concentration more than or in range of 23Kb band of Lambda marker that indicated their molecular weight from 38.8ng to more than 95.4ng in the tested samples (Figure 2 and 3). Distinct band with very less smearing was observed on gel image (Figure 2 and 3). Lane labelled HB and CB showed approximate concentration of DNA >95.4ng and the FB lane DNA concentration was in between 38.8ng to 95.4ng (Table 1).

Table 1: Quantitative analysis of extracted DNA by Lambda DNA marker (NEBN3012S)

DNA marker (NEB N3012S) Hind III digest(Stock concentration500ng/µl, 1µg loaded)	(Working 20ng/µl)	Honey Bee	Carpenter Bee	Flour Beetle
23,130bp(477ng)	95.4ng	>95.4ng	>95.4ng	<95.4ng >38.8ng
9416bps (194ng)	38.8ng			
6557bps (135ng)	27.0ng			
4316bps(90ng)	18.0ng			
2322bps (48ng)	9.60ng			

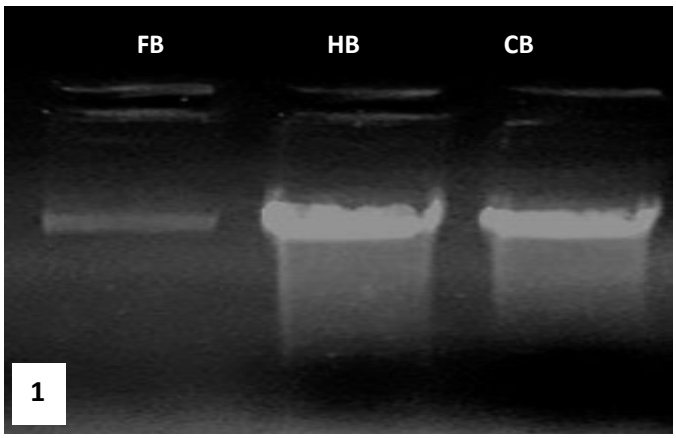
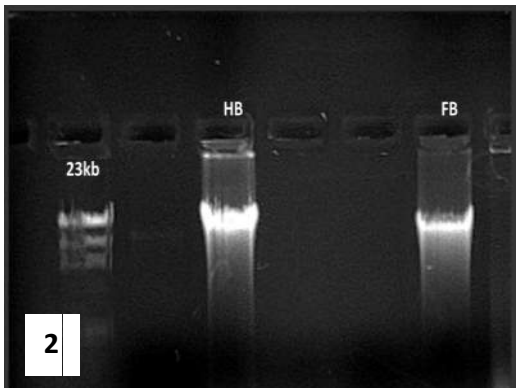


Figure 1: Agarose gel image of genomic DNA samples Flour beetle, FB (*Tribolium* sp.); Honey bee, HB (*Apis mellifera*); Carpenter bee, CB (*Xylocopa* sp.)



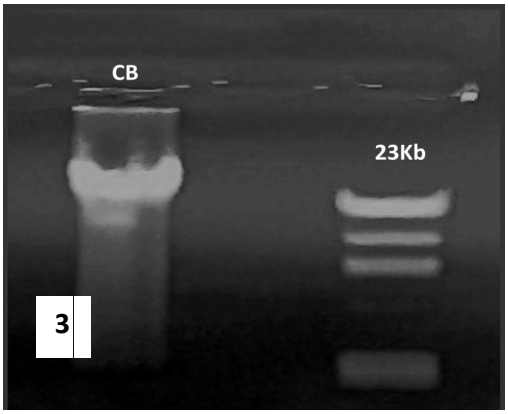


Figure 2 and 3: Genomic DNA of Honey bee (HB), Flour beetles (FB) and Carpenter Bee in 0.8% agarose gel in 1X TAE buffer at 80V for 20minutes by Lambda DNA (NEB N3012S) Hind3 digested marker (23Kb to 2kb)

Nucleic acid absorption at 260nm by spectrophotometer determined the quantity of DNA of the three samples (Table 2). The measured value of O.D at 260nm was 0.98, 1.02, 0.75. Similarly, the O.D value at 280nm was 0.42, 0.58 and 0.39 for samples HB, CB and FB. The concentration was found to be 4900, 5100 and 3750 ng/ul and DNA purity determined by the absorbance ratio at O.D 260/280 nm was 2.33, 1.76 and 1.92.

Table 2: DNA quantification by spectrophotometer, purity checked by $O.D_{260/280}$

Sample	Absorbance at 260nm ($O.D_{260}$)	Absorbance at 280nm ($O.D_{280}$)	Absorbance at 260/280 ($O.D_{260/280}$)	DNA concentration $O.D_{260}$×50ng/µl×100
Honey bee	0.98	0.42	2.33	4900
Carpenter bee	1.02	0.58	1.76	5100
Flour beetle	0.75	0.39	1.92	3750

DISCUSSION

The modified CTAB with beta-mercaptoethanol represented an efficient method of DNA extraction from insect's tissue. We were successful in isolating good quality of DNA by CTAB method. The extraction was performed at room temperature to prevent DNA degradation at higher temperature.

CTAB has been proved to be most essential constituent of lysis buffer for isolating good quality of DNA. Gel electrophoresis revealed a single, high molecular weight DNA band with little evidence of shearing and absence of RNA contamination. Together with, using more concentration of NaCl and mercaptoethanol in the lysis buffer enhanced protein degradation due to which less smearing was observed. Beta mercaptoethanol is a strong reducing agent and denatures protein as well as removes phenolic derivatives present in insects. For removing undesirable carbohydrate from DNA preparations the CTAB method has been considered to be the most efficient method (Weiland, 1997).Many other researchers reported modified CTAB method to extract DNA from diverse plants, fungal spores as well as insects. DNA extraction of medicinal plants from CTAB method was reported by Tiwari et al. (2012).

Likewise, high quality DNA was obtained by SDS/ CTAB method from plant and fungi and even from recalcitrant plants that had elevated concentration of polyphenolics and polysaccharides by Niu et al. (2008). Moreover, the CTAB-PVP method was also used for DNA isolation in xylophagous beetles such as Ataxia alpha, Estoloides chamelae and Lissonotus flavocinctus (Cerambycidae), thus approving that this modified method can be also applicable to xylophagous insects (Calderon-cortes, 2010).

To ascertain the quality and suitability of DNA sample for further analysis DNA quantification and purity was checked. For this marker lambda DNA had provided a rapid and sensitive means to estimate DNA quantity. Accordingly, the marker of variable length analysed the approximate DNA molecular weight. Therefore, the molecular mass of a nucleic acid fragment can be resolved by following agarose gel electrophoresis with ethidium bromide staining thus, comparing the intensity of the fluorescence of a fragment of unknown molecular mass with the intensity of a similarly sized fragment of known molecular mass. Correspondingly, bands in HB and CB lane with band size of 23Kb of lambda DNA had molecular weight more than 95.4ng and FB appeared to be less than 23Kb and more than 9Kb had molecular weight in between 38.8ng to 95.4ng. Analysis of gel electrophoresis image confirmed DNA band intensities as CB> HB> FB (Figure 2 and 3).

A good and uncontaminated preparation of nucleic acid, the A260/280 ratio, which denote protein contamination, should be 1.8 to 2.0 (Sambrook and Russel, 2001). Table 2 summarizes the DNA purity range obtained for all sample extracts. A 260/280 ratio in this study was found to be 1.76 and 1.92 for CB and FB samples revealing pure genomic DNA however, ratio of 2.33 in HB sample revealed slight contamination of phenol or chloroform. Absorbance at 260nm illustrated DNA concentration of 1.02> 0.98> 0.75 of CB> HB> FB with concentrations of 5100, 4900 and 3750 ng/ul. Therefore, A260nm determined the quantization levels of the samples and the ratio of A260/A280nm determined the quality. Hence, traditional CTAB method with slight modification with proper handling has been proved to be suitable for isolation of genomic DNA that could be considered further for PCR and key to identify wide range of different insects.

ACKNOWLEDGEMENT

We are grateful to Department of Zoology, Babasaheb Bhimrao Ambedkar University for providing research facilities to accomplish this research.

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