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Enhanced DNA Extraction Protocol for Larvae and Adult Specimens of *Clostera cupreata* (Butler) (Lepidoptera: Notodontidae): A Key Leaf Defoliator of Poplar Trees

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Authors' contributions

This work was carried out in collaboration among all authors. Author JK conceptualized the study, performed the methodology, searched for resources, did visualization, wrote and prepared the original draft of the manuscript, wrote, reviewed and edited the manuscript. Authors AK and RKM conceptualized the study, searched for resources, supervised the work, wrote, reviewed and edited the manuscript. Authors JK and AS did formal analysis, data curation and investigation. All authors read and approved the final manuscript.

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ABSTRACT

The CTAB DNA isolation protocol for both larval and adult specimens of *Clostera cupreata* has optimized with modifications included adjusting the concentrations of EDTA, SDS, and NaCl; substituting liquid nitrogen with extraction buffer for sample homogenization; and extending the incubation, mixing, and precipitation time period. This modified protocol ease of use and efficiency allow for the quick extraction of high-quality, high-yield entire genomic DNA using common reagents and equipment used in molecular laboratories. This will enable the documentation of genetic variability based on biotypes, facilitating studies on the emergence, distribution, and population dynamics of *C. cupreata*. To preserve the integrity of the extracted DNA, stringent measures are implemented to prevent contamination from various biological by-products, including proteins, salts, phenols, and polysaccharides. We have verified the quality and purity of the obtained DNA samples through standard quality assessments. The A260/A280 ratios consistently fall within the range of 1.80 to 1.88, providing further validation of the integrity and suitability of the isolated DNA by performing polymerase chain reaction analysis using mitochondrial COI primer. This approach is suitable for a wide range of molecular applications. Our extraction protocol yields high-quality DNA that may be used for sophisticated molecular processes like gene cloning, conventional polymerase chain reaction for genotyping, next-generation sequencing and barcode synthesis. Furthermore, this approach has potential as a preventive diagnostic tool for detecting invasive lepidopteran larval stages.

Keywords: Clostera cupreata; DNA extraction; PCR; CTAB; isolation protocol.

1. INTRODUCTION

In North-Western India, *Populus deltoides* (Family: Salicaceae) commonly called poplar are prone to leaf defoliation caused by *Clostera cupreata* (Lepidoptera: Notodontidae) a major insect pest in the poplar growing region [1,2]. More than 25% tree defoliation caused by this species is known for significant reduction in the growth of poplar tree [3] and the incidence of *C. cupreata* based on percent damaged leaves and pupae of *C. cupreata* having its peak (46.48%) in September [4]. The larval stages of this pest are gregarious in nature and are voracious feeders. They scrap the leaf epidermis and skeletonize the leaves. The older larvae (4^{th} and 5^{th} instar) are solitary in nature and eat away all the tissues of the leaves leaving behind only the veins [5]. The early detection and monitoring of significant pests are vital in safeguarding valuable agroforestry commodities from infestation and dissemination, While the classification of insect species typically depends on their physical and morphological traits, identifying specific species becomes problematic due to the presence of common characteristics that create similarities between them [6,7]. The molecular techniques including DNA markers plays a vital role in precise species identification, genetic diversity analysis, establishment of DNA barcoding libraries and serving as complementary evidence to the traditional morphology-based classification methods [8]. A critical step in molecular biology is optimizing the DNA extraction protocol because the quality and quantity of the extracted DNA impact subsequent applications such as polymerase chain reaction (PCR) and other amplification-based methods. Therefore, it is essential to have a reliable, easy-to-use, fast and inexpensive DNA extraction protocol to generate accurate and high-quality DNA [9-11].

For the majority of insect species, achieving high-quality and high-yield DNA poses a significant challenge due to the presence of inhibitory compounds in the extraction process. These compounds typically stem from the processed biological sample itself, including polysaccharides or phenols, as well as from the chemicals employed to render the DNA accessible, such as CTAB [12]. However, there is a limited amount of research that compares extraction methods to determine the most suitable option for individual species or families [13]. It is difficult to dissect these insects in order to extract DNA since their digestive systems contain plant phenolics and tannins, especially when they are at larval stage. In later molecular analysis, these phenolics and other secondary compounds have the potential to damage DNA directly or obstruct enzymatic activity. This is particularly problematic for procedures like barcoding, creating genomic libraries, or performing Southern blot analysis, which depends on enzymes like Taq polymerases or restriction endonucleases [14,15]. While the

CTAB-based DNA extraction method is widely employed, it may not always effectively eliminate all phenolics from DNA preparations. Therefore, antioxidants such as PVP is commonly used to address issues related to phenolics [15]. The cetyltrimethylammonium bromide (CTAB) method has proven to be effective in extracting DNA from various insect species, including ants, bees, butterflies and mosquitoes.

In addition, several commercially available DNA extraction kits are recommended for insect DNA isolation such as DNeasy Blood & Tissue Kit from Qiagen, DNAzol reagent and Puregene Kit [10,16,17]. However, these kits are not appropriate for isolating DNA from certain insect species, especially those containing high levels of polysaccharides, polyphenols, proteins and other cellular by-products. Additionally, they are expensive per sample and yield lower amounts of DNA compared to standardized extraction protocols [10]. Previous research opted a cost-effective commercial kit for DNA extraction due to the lack of a standardized extraction protocol for the studied samples. However, the obtained samples often exhibited subpar quality or degradation. To enhance DNA quality, researchers turned to the DNeasy Blood & Tissue Kit from Qiagen, which is pricey and time-intensive. Nevertheless, the high cost and limited availability of these kits often pose challenges and obstacles in research and laboratory work [9]. For DNA extraction from a wide range of organisms, methods utilizing sodium dodecyl sulfate (SDS) and CTAB are frequently employed. These techniques yield DNA that is suitable for use as a template in PCR, enabling the generation of distinct molecular markers [18]. The CTAB protocol has been effectively utilized for DNA extraction from several insect species, including *Apis mellifera, Trichogramma evanescens*, *Chrysoperla carnea*, *Tribolium castaneum*, *Cicindela campestris*, *Lymantria dispar*, *Ephestia kuehniella* and *Plodia interpunctella* [19]. It is important to note that the success of DNA extraction can be affected by various factors, such as the type of tissue, the age of the sample and the presence of inhibitors [20,21]. Therefore, it is recommended to optimize the protocol for the specific insect species and tissue type being used.

C. cupreata poses a significant threat to *P. deltoides* in North-Western India, causing severe leaf defoliation and reduced tree growth. Early detection and accurate identification are vital for effective management. While traditional identification relies on morphological traits, molecular techniques, such as DNA markers, offer greater precision. However, extracting high-quality DNA from *C. cupreata* is challenging due to inhibitory compounds like phenolics and tannins, which can compromise DNA extraction protocols such as CTAB. Although commercial kits exist, they are costly and often produce insufficient DNA. To address these limitations, we aim to develop an optimized, cost-effective DNA isolation protocol specifically tailored for *C. cupreata* using larval and adult tissues. Our modified CTAB protocol is designed to yield high-quality, high-yield genomic DNA with minimal contamination, providing a reliable tool for molecular studies on this pest, including research in pest management, population dynamics, and genetic diversity.

2. MATERIALS AND METHODS

2.1 Sample Collection and Preparation

The larval stage of the *C. cupreata* was collected from poplar fields from Haridwar district (29°54’9” N and 78°0’18” E) of Uttarakhand state, India and reared separately on natural diet under controlled condition (28 ± 2 °C temperature and 65 ± 5% relative humidity) at Forest Entomology Discipline, Forest Research Institute, Dehradun, India upto adult emergence in the laboratory. Laval and adult samples were promptly placed in 2 ml microcentrifuge tube of absolute alcohol and stored at -20˚C until extraction procedure was carried out. To efficiently extract DNA from *C. cupreata*, we initially employed two protocol: (P1) the "Salting out method" described by [14] and the (P2) "CTAB method" outlined by [22]. Following testing, the CTAB method provided the most satisfactory results. Subsequently, we selected and refined this method for DNA extraction from *C. cupreata* by adjusting the concentrations of ethylenediamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS) and sodium chloride (NaCl). Here, we present only the successful DNA isolation procedure, along with the modifications implemented and the purification steps undertaken.

2.2 Solutions, Reagents and Supplies

The reagents and solutions necessary for the experiment were obtained from reputable commercial suppliers, ensuring their quality and accuracy. The stock solutions employed included UltraPure™ 0.1 M Tris-HCl (pH 8.0) from Thermo Fisher Scientific, UltraPure™ 10M EDTA; pH 8.0 from SRL, UltraPure™ SDS from Thermo Fisher

Scientific, 20 U/mg Proteinase K from Sigma-Aldrich, 10% CTAB from HIMEDIA, 5M Ammonium acetate (NH_4Ac) from HIMEDIA, Isoamyl-alcohol, chloroform, absolute ethanol and 2-propanol from Merck. These reagents were carefully selected and met the required standards for molecular biology applications. Glassware used are of Borosil and Rivera, and plasticware from Tarsons and Eppendorf was also used.

2.3 Optimized Protocol for DNA Extraction

- To begin the DNA extraction process, heat up an extraction buffer solution with a pH of 8 that contains 0.1mM Tris-HCl, 10mM EDTA and 2% SDS in water bath at 60ºC for 15 minutes.
- The process of extracting DNA from larval and adult stage samples (approx. 0.50 mg) involves drying at room temperature for 1 hour and homogenized it directly using 2ml preheated extraction buffer.
- Once the homogenization was complete, it was carefully transferred into a 2ml microcentrifuge tube along with 2 µl of proteinase K. Subsequently, the microcentrifuge tube was placed for incubation in water bath at a temperature of 58°C for 60 minutes. Throughout the incubation period, the microcentrifuge tube was gently inverted several times to facilitate thorough mixing.
- After the completion of incubation, add 140 µl of 5M NaCl and 75 µl of 10% CTAB in microcentrifuge tube having sample, Subsequently, the microcentrifuge tube was incubated at 65°C for one hour to facilitate the next step.
- Once the incubation is complete, the samples are left to cool at room temperature for 10 to 15 minutes. Then, 700µl of chloroform-isoamyl alcohol (24:1) was added to the mixture, which is then mixed thoroughly for 15-20 minutes until emulsion is formed.
- Then, formed emulsion is centrifuged at room temperature at 14,000 rpm for 10 minutes. The resulting supernatant or aqueous phase is then carefully transferred to 1.5 ml sterile centrifuge tubes in to which 225 µl of 5M ammonium acetate was added.
- Incubate the sample on chilled ice for 30 min, then centrifuged at 14,000 rpm for 20 min. at 4°C.
- On completion of centrifugation, the separated supernatant was transferred to a new 1.5 ml microcentrifuge tube.
- 500 µl of chilled isopropanol was added to the supernatant & mixture is gently inverted and mixed, and then incubated overnight at -20 ºC.

Note: The longer the chilled incubation, the more the precipitation.

- Next, the mixture is centrifuged at 14,000 rpm for 15 minutes at 4ºC to form pellet. The supernatant is then discarded and the resulting pellet was washed with 500 µl of chilled 80% ethanol and centrifuged at 15,000 rpm for 15 minutes at 4°C.
- Vacuum dry the pellet on thermo block for 15 minutes at 37 ºC.

Note: Make sure that there is no residual ethanol, this is very critical especially if the DNA is to be used directly for PCR. Over drying should also be avoided as it makes the pellet difficult to resuspend.

- Add 100 µl of TE buffer (consisting of 10mM Tris-HCl, 1mM EDTA) of pH 8 which has been autoclaved, to dissolve the precipitate.
- The DNA obtained from the extraction process was preserved at a temperature of -20 °C until it was required for subsequent use.

2.4 Quality and Quantity Analysis of Genomic DNA

To assess the quality of the DNA samples, electrophoresis was performed using a 0.8% agarose gel with 1X TBE buffer, the gel was stained with ethidium bromide (0.5 µg/ml) and subjected to electrophoresis at 120V for 50 minutes. Following gel electrophoresis, the bands were observed using a gel documentation imaging system (GelDoc-It Imaging system, UVp model LMS-20E, Upland, USA) and DNA purity was determined by measuring the absorbance ratio at A260/A280 nm using a UV-Visible spectrophotometer, and the DNA concentration was measured in ng/µl.

2.5 CO1 Gene Amplification

The integrity and purity of extracted DNA were assessed through PCR amplification using universal primers LCO1490

(5'GGTCAACAAATCATAAAGATATTGG3') and HCO2198 (5' TAAACTTCAGGGTGA CCAAAAA ATCA 3'), targeting a 680 bp fragment of the cytochrome oxidase I (COI) gene known as the barcoding region. For PCR amplification, a 15 µl reaction mixture was prepared, consisting of 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.2 µM of each primer, 1 unit of Taq polymerase (M/S Bangalore Genei, India), and 15 ng of template DNA. The amplification process was carried out using the Eppendorf Mastercycler® nexus, following a program that included initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 48.3°C for 1 minute, and extension at 72°C for 1 minute with final extension step at 72°C for 10 minutes. The amplified products were visualized by running them on a 2% agarose gel. After conducting gel electrophoresis, the bands were visualized using the GelDoc-It Imaging system (UVp model LMS-20E, Upland, USA). The Gel Extraction kit (G-Bioscience) was employed for extracting and purifying the desired-sized samples from the agarose gel. Subsequently, these purified samples underwent sequencing (both strands) through the services provided by Barcode Bioscience Pvt Ltd, Bangalore, India.

3. RESULTS

In the initial phase of our study, we extracted DNA from *C. cupreata* using the Salting out method [14] resulting in DNA yields ranging from 120 to 142 ng/µL and the DNA purity ratios (A260/A280) varied from 1.26 to 1.40. Additionally, employing the CTAB method proposed by [22] yielded DNA concentrations ranging from 200 to 240 ng/µL, with DNA purity ratios (A260/A280) ranging from 1.52 to 1.57. This preliminary assessment indicated that the Salting out method was not suitable for extracting DNA from *C. cupreata* tissues, possibly due to the presence of inhibitory compounds that were not effectively removed during the washing steps and the absence of efficient enzymatic lysis. Based on the qualitative analysis of DNA using gel electrophoresis on 0.8% agarose gel (Fig. 1), we made modifications to the previously reported CTAB method in order to enhance the DNA quality. These modifications involved adjusting the concentrations of EDTA, SDS and NaCl, substituting liquid nitrogen with extraction buffer for sample homogenization (Table 1), prolonging incubation, mixing and precipitation time period. These modifications collectively contributed to the improvement of DNA purity.

The utilization of the modified CTAB protocol in this study resulted in the extraction of DNA with exceptional quality, exhibiting no signs of degradation or smearing from both larvae and adult specimens of *C. cupreata*. Importantly, the effectiveness of the DNA extraction and purification process was verified through gel electrophoresis, revealing distinct and well-defined bands on the 0.8% agarose gel (Fig. 2).

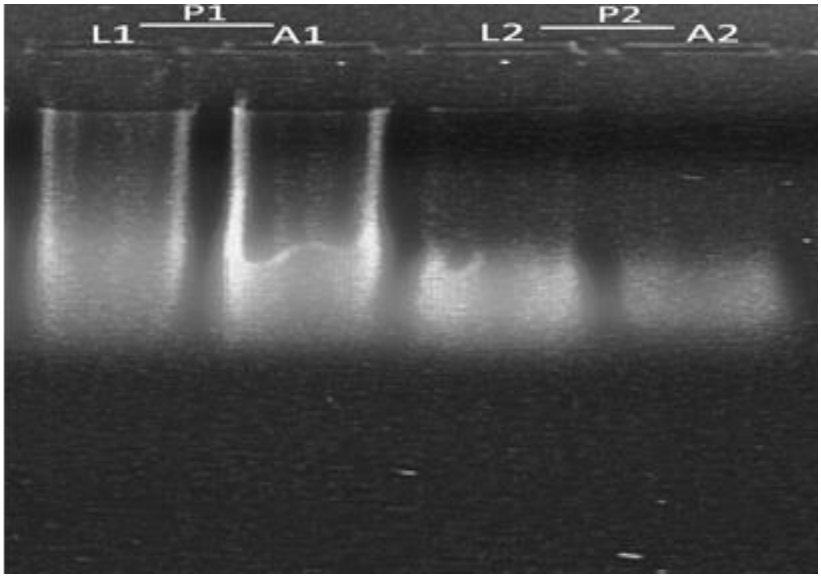

Fig. 1. Extracted genomic DNA from two DNA isolation methods: CTAB method (P1) and Salting out method (P2). L; Larva, A; Adult specimen

Table 1. Composition of extraction buffer using in different protocols

Chemicals and Reagents	CTAB protocol (Moeller et al. 1992)	Modified CTAB protocol
Homogenization	Liquid Nitrogen	Extraction Buffer
Tris-HCl (pH 8)	0.1 M	0.1 M
EDTA	10 mM	20 mM
SDS	2%	10%
Proteinase K	0.2 mg/ml	0.2 mg/ml
NaCl	5 M	2 M
CTAB	10%	10%
NH_4Ac	5 M	5 M
PEG	30 %	NA

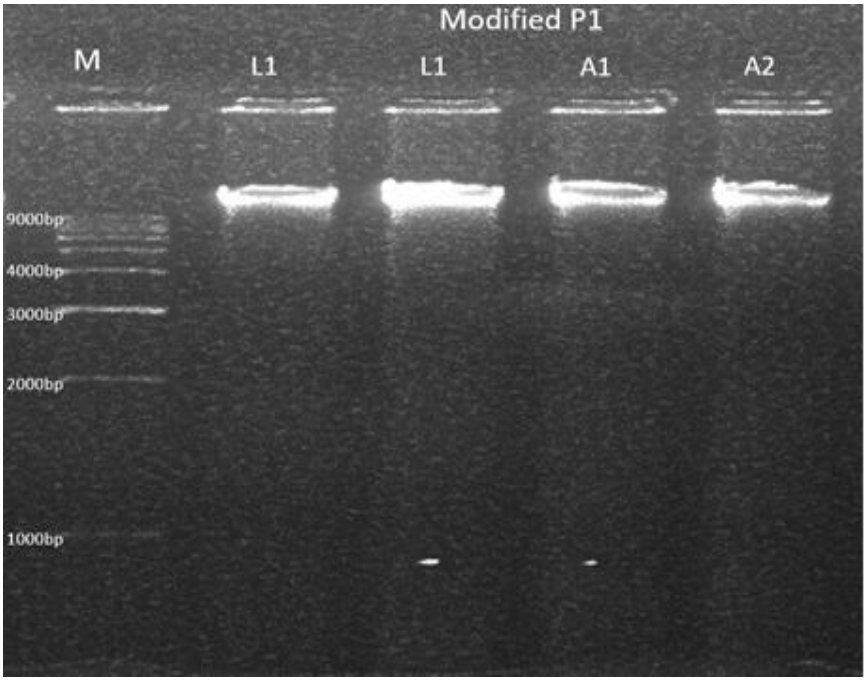

Fig. 2. Extracted genomic DNA from two larvae (L1–L2) and two adult (A1–A2) specimens of *C. cupreata* using the modified CTAB method (P1), separated by electrophoresis on a 0.8% agarose gel with a 1 Kb ladder as a reference

The optimized protocol yielded DNA concentrations ranging from 250 to 320 ng/µL, with purity ratios (A260/A280) ranging from 1.80 to 1.88, indicating a significant quantity and high purity of the extracted DNA. These findings confirm that the modified DNA isolation protocol generates a substantial amount of high-quality DNA suitable for a variety of PCR reactions and other molecular techniques. To confirm the performance of the polymerase enzyme and ensure the purity and integrity of the extracted DNA, a PCR amplification targeting a 680-bp segment of the Cytochrome oxidase I (COI) gene was conducted using 15 ng of the extracted DNA as a template. The results of gel electrophoresis for the COI amplicon generated through PCR demonstrated clear bands (Fig. 3).

The visibility of the COI amplicon confirms the excellent quality of the extracted DNA, validating its successful amplification using the PCR method. The chromatogram displayed neat peaks, evenly distributed and each sporting a single colour (Fig. 4). Background interference was minimal, with only occasional slight noise, but it didn't hinder peak identification. Sequencing the fragments and matching them against the NCBI database revealed similarities to known sequences from other *Clostera* species experiments. These results demonstrate that the modified-CTAB DNA isolation protocol yields DNA of high quality and quantity, suitable for numerous PCR reactions and other DNA manipulation techniques.

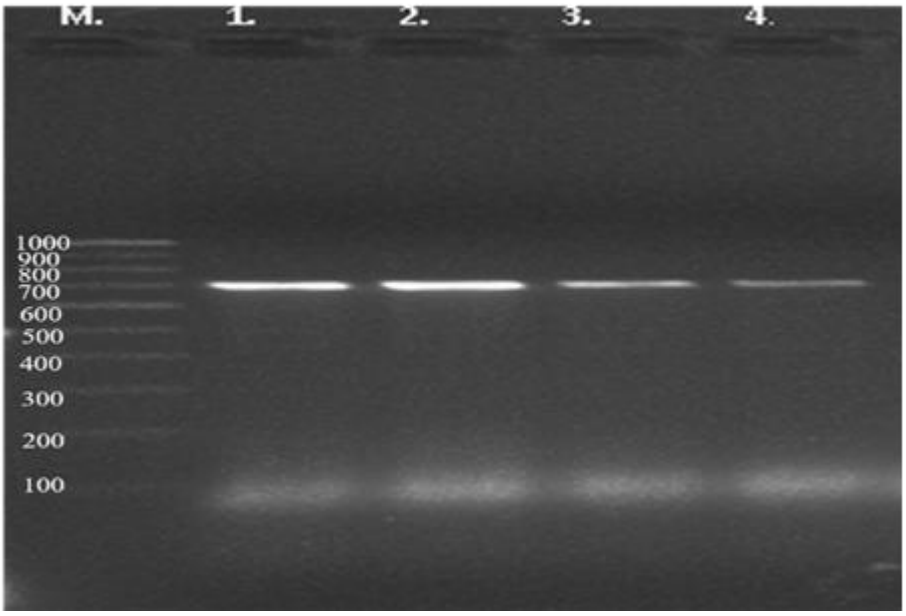

Fig. 3. A PCR profile of larvae (1-2) and adult (3-4) specimens of *C. cupreata* generated by the universal primer mitochondrial cytochrome oxidase I (COI). M:100 bp DNA ladder

4. DISCUSSION

The field of entomology encounters obstacles when it comes to distinguishing and characterizing similar species due to their notable morphological similarities [23]. To addressing these complexities necessitates the development and implementation of dependable methodologies that can accurately differentiate and characterize species [24]. Molecular biomarker methodologies have been advocated as a valuable adjunct to traditional morphological classification within the scientific domain. Nonetheless, the presence of impurities in the isolated DNA can exert a deleterious influence on the efficacy of downstream applications [25], including polymerase chain reaction (PCR) and enzymatic modification of DNA, consequently leading to the potential degradation of DNA during storage [26]. Thus, it is paramount to utilize a meticulously optimized DNA extraction protocol that generates DNA of superior quality, integrity and purity.

In recent times, there has been a notable drive to develop highly efficient protocols for DNA extraction from insects that are characterized by their rapidity, cost-effectiveness and ecological compatibility. These advancements aim to minimize the associated hazards and risks typically associated with the extraction process, promoting safer and more sustainable practices [27,28]. Nonetheless, the presence of secondary compounds, including polyphenols and polysaccharides in insect tissues necessitates the optimization of specific extraction methods tailored to each insect species and tissue type. Previous investigations have explored the impact of different concentrations of β-mercapto ethanol and NaCl on the extracted DNA's quality, as these compounds facilitate the removal of polyphenols and polysaccharides during the DNA extraction process [15,29]. Several techniques have been devised for the extraction of DNA from insects, although they often involve time-consuming steps for the separation of impurities and contaminants. Furthermore, the affordability of commercial kits can present a barrier when compared to traditional methods [9]. The yield and purity of extracted DNA can also vary depending on the insect species and the specific extraction protocol used [14,30]. Therefore, the aim is to develop a more efficient DNA extraction method while still maintaining high yield and quality.

According to our suggested protocol, the inclusion of EDTA initiates the disruption of the cell wall, facilitating the release of nucleic acids [31]. The addition of Tris-HCl serves the purpose of pH equilibration, bringing it close to 8.0. Sodium chloride, on the other hand, effectively aids in the extraction of nucleic acids from polysaccharide compounds [32,33]. SDS

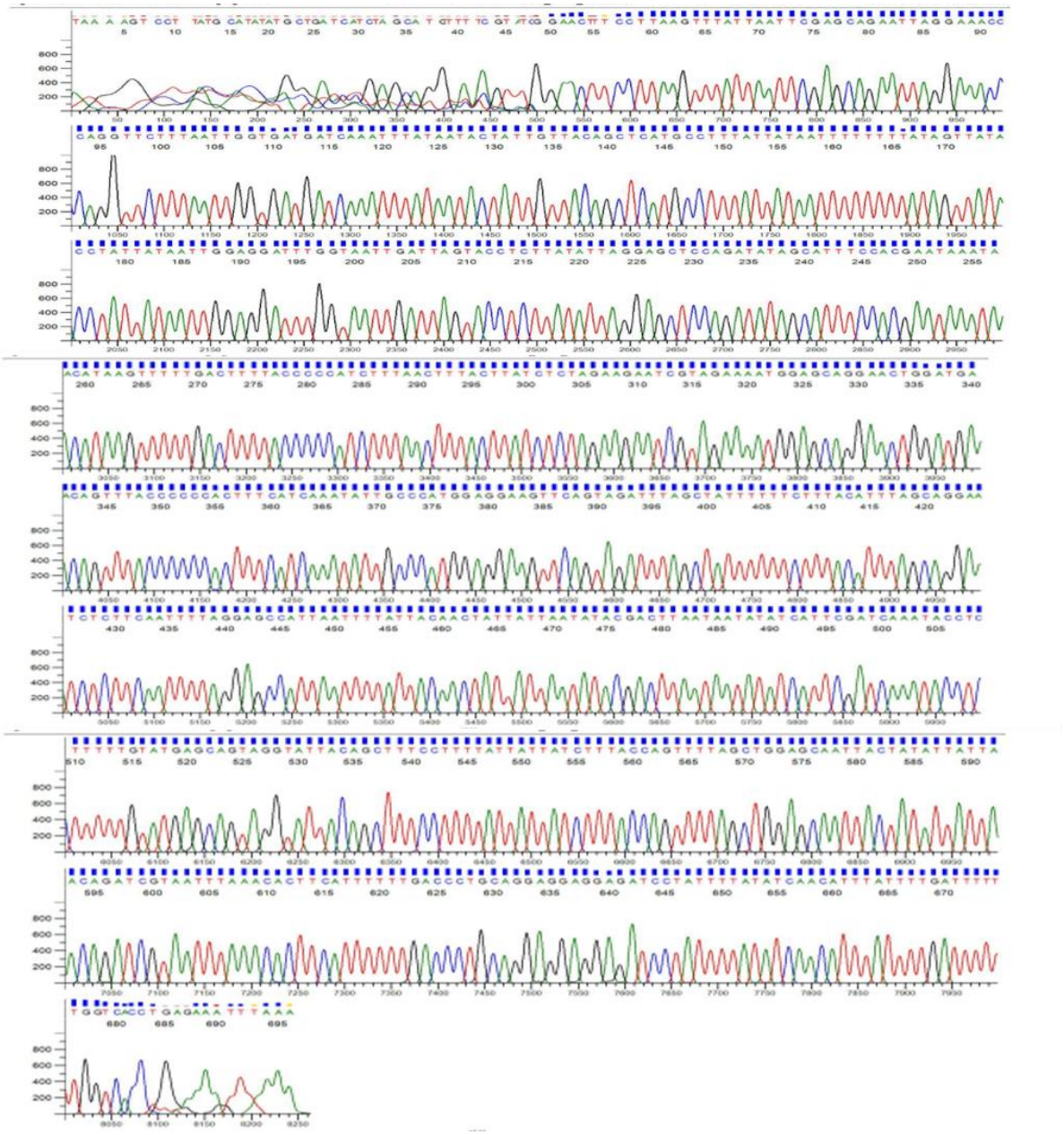

Fig. 4. Resulting chromatogram of the COI region sequencing

functions as an anionic detergent, facilitating the disruption of the cell membrane and nuclear envelope. It also acts to neutralize negative charges on amino acids. The presence of CTAB in the buffer solution has been demonstrated to yield DNA of exceptional quality by targeting the lipid layer of the membrane, effectively eliminating proteins. Proteinase K plays a pivotal role in protein digestion and the removal of contaminants during nucleic acid preparation. NaCl aids in the detachment of proteins bound to DNA, while ammonium acetate precipitates proteins. The use of chloroform-isoamyl alcohol for washing enhances protein precipitation and reduces polysaccharide content. Additionally, performing chloroform washes at the outset of DNA isolation eliminates debris and proteins. Agitation with chloroform-isoamyl alcohol in a shaker promotes cell wall disruption, leading to improved enzymatic lysis, reduced DNA fragmentation, and a heightened DNA yield. These findings strongly indicate that the inclusion of CTAB and its modifications not only enhances DNA yield but also effectively eliminates potential contamination from lipids, proteins and other cellular compounds that may interfere with

downstream DNA applications. Our results align with a study [34] that reported a DNA extraction protocol for green lacewings (*Chrysoperla carnea*) using 2% CTAB and 1M NaCl in the extraction buffer [27]. A simple and high-throughput method for DNA extraction from insects was also presented, though it included the use of 10 mg/ml proteinase K. This approach streamlines the extraction process, making it more efficient and accessible for large-scale studies. The use of proteinase K at this concentration ensures effective digestion of proteins, facilitating the release of high-quality DNA suitable for subsequent molecular analysis.

5. CONCLUSION

Developed protocol successfully isolates high-quality DNA from *C. cupreata* tissues, yielding intact DNA without the need for liquid nitrogen, phenol, or enzymatic agents such as RNAase. The process consistently produces DNA samples free from inhibitors, making them ideal for PCR amplification and sequencing. Additionally, the protocol is effective for both larvae preserved in absolute alcohol and adults stored at -20°C. With a total duration of 6 hours, including a more than 3 hours incubation period, this method offers a practical and efficient approach for detecting *Clostera* species and allowing other tasks to be conducted simultaneously by researchers. The necessary chemicals, consumables, and materials are commonly available in most laboratories, making the protocol easily adoptable for preliminary researchers working with a large number of pest samples to study polymorphism or barcoding DNA within a set timeframe.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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