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Original Research

A simplified and optimized protocol for total DNA extraction from insect species: applicable for studying genetic diversity and PCR-based specimen identification via partial amplification of cytochrome oxidase I (COI) gene

Samira Paydar[1], Esra Ucar[2], Peyman Yari[3,4], Seyed Mehdi Safavi[5], Danial Kahrizi[6], Masoud Nateqi[7], Alinaghi Mirmoayedi[8], Kheirollah Yari[*3,9]

[1] Department of Agronomy and Plant Breeding, Razi University, Kermanshah, Iran
[2] Department of Crop and Animal Production, Sivas Vocational School, Cumhuriyet University, Sivas, Turkey
[3] Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran
[4] Department of Medicinal Plants, Institute of Higher Education, Jahad-e-Daneshgahi, Kermanshah Unit, Iran
[5] Department of Agronomy and Plant Breeding, Kermanshah Branch, Islamic Azad University, Kermanshah, Iran
[6] Fertility and Infertility Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran
[7] Department of Plant Protection, Faculty of Agricultural Sciences and Engineering, University of Tehran, Karaj, Iran.
[8] Department of Plant Protection, Razi University, Kermanshah, Iran
[9] Zagros Bioidea Company, Razi University Incubator, Kermanshah, Iran

Correspondence to: kyari@kums.ac.ir, khirollah.yari@yahoo.com

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Abstract: The efficient DNA extraction from insects has been suggested as a critical and main step affecting molecular entomology for taxonomic identification, the establishment of DNA barcoding library and analysis of genetic diversity relationship between insect populations. For successfully apply these molecular techniques, high-quantity and high-quality of the extracted DNA are required. Several protocols for efficient genomic DNA extraction from insects have been developed. In this research, we represent a rapid, reliable and cost-effective method that it is not reliant on poisonous and enzymatic reagents for DNA extraction from insect tissues. Results showed that high quantity and high-quality of the isolated DNA by this method is suitable and can be used directly for PCR, also is enough to do hundreds of molecular reactions. In conclusion, we described a fast, cost-effective, non-toxic and enzyme-free protocol for high yield genomic DNA extraction from green Lacewings (*Chrysoperla carnea*) tissues in basic equipment laboratories.

Key words: DNA extraction method; Optimized protocol; Chrysopidae; Cytochrome oxidase I.

Introduction

In molecular entomology, research on the DNA markers is an effective tool for analysis of taxonomic and phylogenetic relationships among insect populations and also in evolutionary studies (1-3). The classification of insect species is based on the physical and morphological characteristics, but some of the species in this family resemble each other and could not be identified easily (3, 4). Hence, in entomology field, development of molecular techniques such as DNA markers would be beneficial tools for accurate identification of species, detecting the genetic diversity, the establishment of DNA barcoding library and to find a corroborative evidence for the traditional morphology-based classification (1, 5, 6). Also, in molecular DNA technology, optimization of DNA extraction protocol is a crucial step, because the quality and quantity of the extracted DNA affect the results of subsequent applications such as Polymerase Chain Reaction (PCR) and other amplification-based methods (7-11). For the first time, DNA extraction was performed in 1869 by the young Swiss physician Friedrich Miescher. Several modified protocols for DNA extraction from insects have been developed in the past twenty years. Nevertheless, to our knowledge, there isn't a common and an optimized protocol for genomic DNA extraction from different insect's tissues. So the method for DNA isolation with high quality must be optimized to different insect species even to different insect tissue. Today's, most of the current protocols for DNA extraction from insects tissues are based on methods of CTAB, phenol-chloroform, 71 SDS/proteinase K or commercially available kits (7, 12). These available protocols are time-consuming, laborious with using hazardous chemical reagents like phenol and enzymes such as Proteinase K for DNA deproteinization. Although, commercial insects DNA isolation kits are fast and safer, usually are very expensive, low yielded and also requiring expensive types of equipment in the laboratory and may not available everywhere (9). Laboratory experts and researchers need to relatively fast, inexpensive and high throughput DNA extraction protocol (13). Therefore, an optimized and efficient protocol for DNA extraction from insect tissues is an essential step to follow up a reaction in molecular entomology technology. The aim of this research was focused on obtaining a novel optimized protocol for the efficient DNA extraction from insects (Green Lacewing

species) without using hazardous chemical reagents and typical enzymes we represent a rapid, reliable and cost-effective method that it is not reliant on poisonous and enzymatic reagents for DNA extraction from insect tissues.

Materials and Methods

The Green Lacewing (*Chrysoperla carnea*) species as the sample of insect's species were collected from Kermanshah province (33°36′ - 35°15′ W, 45°24′ - 48°30′ E), Iran. Insect species were stored in 70% ethanol until use.

Reagents and chemicals

All of the reagents and chemical materials including, Tris base, NaCl, EDTA, Chloroform, CTAB, Agarose were purchased from Merck Company (Germany).

Buffer A solution: 50 mM Tris HCl, 2 % CTAB, 1M NaCl, (pH 7.6), 2% β-mercaptoethanol, 10 mM Na_2EDTA (14).

Optimized protocol for DNA extraction from insects' tissues

1. One hundred mg of tissue (usually thoracic muscle, wings, head and leg muscles) was placed in a 2-ml microtube and tissues was ground with micropestle.
2. One ml of buffer A and 100 µl of SDS (10%) were added into a microtube and incubated in water bath at 70 °C for 60 min, the microtube inverted several times for mix during incubation.
3. The microtube was centrifuged (Sigma) at 5000 rpm for 8 min, and the supernatant was transferred into a new 2-ml microtube.
4. The microtube chilled in ice for 3 min then 400 µl of chloroform and 400 µl of saturated NaCl (5M) were added and gently were shacked for 30 sec, centrifuged at 5000 rpm for 10 min at 4°C.
5. The upper phase (~1 ml) transferred into a new 2-ml microtube, and an equal volume of cold absolute ethanol was added.
6. The tube was gently mixed; in this step, the genomic DNA appeared as a white skein.
7. Again, the microtube was centrifuged for 10 min at13000 rpm at 4°C.
8. The supernatant was discarded and the pellet was washed with 750 µl of chilled 70% ethanol and centrifuged at 10000 rpm for 10 min.
9. The supernatant was discarded and let the pellet to dry at room temperature.
10. The pellet was dissolved in 50-100 microliter of ddH_2O or TE buffer
11. Quantity and quality of extracted DNA were quantified with gel electrophoresis and Nanodrop
12. The DNA sample was stored at -20°C until use.

Quantification of extracted DNA

The concentration and yield of extracted DNA were assessed spectrometrically using Nanodrop (Thermo) by measuring the absorbance at a wavelength of 260 nm. Also, quality of extracted DNA was checked with the measuring the OD 260/280 and OD 260/230 absorbance ratios, and the probability of DNA degradation was evaluated by loading 6 µl of extracted DNA using electrophoresis on 0.8% agarose gel containing DNA Safe Stain (Invitrogen) (15, 16).

COI gene amplification

A 850 bp fragment of *cytochrome oxidase* I *(COI)* as a representative of the constitutive gene was amplified with the following primers:

Forward: 5′ CAACATTTATTTTGATTTTTTGG 3′

Reverse: 5′ TCCATTGCACTAATCTGCCATATTA 3′

PCR was performed using Master Cycler Gradient-Eppendorf (*Pro S* model, Germany) with 20 pmol of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1U Taq polymerase and 2.5 µl of 10x PCR buffer, 200 ng of extracted DNA in a final volume of 25 µl. The PCR thermal cycling parameters were: 1 cycle at 94 ˚C for 5 min, followed by 35 cycles at 94 ˚C for 1 min, 1 min annealing at 50 ˚C and extension at 72 ˚C for 1:15 min and then after the last cycle was continued for 10 min at 72 °C as final extension. PCR product was monitored using electrophoresis on 1% agarose gel containing DNA Safe Stain and the predicted size of amplified DNA was confirmed using 100 bp molecular weight marker.

Results

Figure 1 shows the integrity of extracted DNA isolated with our protocol. There is no smear or DNA degradation during extraction. Also, a band of the extracted DNA with high molecular weight was quite sharp in 1 % agarose gel electrophoresis (Figure 1). The yield of this optimized method was high and varied 1000-2000 ng/µl for green lacewing tissue. No contamination detected in all extracted samples. The purity of the extracted DNA was assayed with spectrophotometric analysis

Figure 1. Agarose gel (1%) electrophoresis for isolated total genomic DNA. The extraction protocol was performed with the current optimized protocol (Lane 1-2).

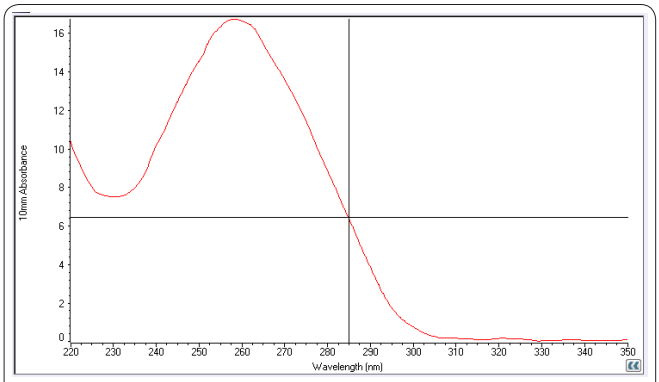

Figure 2. UV–Visible absorption spectrum of the purified DNA.

(Nanodrop, Thermo Model); our results showed that the A260/A280 ratio was from 1.80 to 2, and the 157 A260/A230 ratio was >2.

These results indicated that the DNA isolation with this protocol enables to obtain high quality and quantity and without proteins or salts contamination (Fig. 2).

This high quantity and the quality of the extracted DNA by this method is enough to do hundreds of PCR reactions and also to be used in other DNA manipulation techniques. Therefore, based on spectrophotometric analysis results, the efficiency of the current protocol for DNA extraction from Green Lacewing was confirmed.

Samples with crude DNA generally contain potent PCR amplification inhibitors. To polymerase enzyme performance testing and to 166 make sure whether extracted DNA is intact and naked, *Cytochrome oxidase I* (*CO I*) gene (as a representative of constitutive genes) was amplified. Figure 3 shows the result of agarose gel electrophoresis for 850-bp COI gene fragment that amplified using PCR. About 200 ng of extracted DNA was used as a template in the PCR reaction. The sharpness of COI gene shows that the extracted DNA has high quality and can be amplified by PCR method.

Therefore, based on spectrophotometric analysis results, the efficiency of the current protocol for DNA extraction from green lacewing was confirmed.

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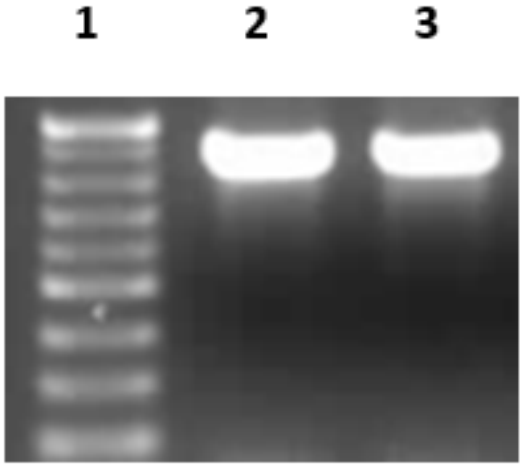

Figure 3. gel electrophoresis on 1% gel electrophoresis for *CO I* gene that amplified by PCR method. Lane 1 shows 100 bp molecular DNA size marker, lane 2 and 3, show 850 bp product bands.

Discussion

In molecular entomology, some of the species due to the high degree of similarity could not be identified based on morphological characters and causes an extra challenge for the evolutionary entomologist to identify and determine of diversity among similar species (5, 17). Thus, new reliable methods are needed for identification of their species. Application of molecular biomarker approaches offers a useful complementary tool for species differentiation with greater reliability and to find a corroborative support for morphologically-based classification of previous authors (18).

DNA extraction has been suggested as the main step affecting molecular DNA technology such as PCR and PCR based methods, therefore, researchers have been used several modified protocols for efficient DNA extraction from animal and plant samples (8, 12, 19-21). Impurity of isolated DNA can reduce the efficiency of downstream applications, for example, PCR and PCR based methods, enzymatic modification of DNA and also it could result in DNA degradation during storage (22, 23). Therefore, for successfully apply to any molecular techniques, an optimized protocol for extraction of pure, intact with high-quantity and high-quality DNA is required (6, 21, 24). In recent decades, attempts in the success of rapid, less hazardous, inexpensive and optimized protocol for DNA extraction with high quality from insects were increased (2, 25, 26). Nevertheless, because of the presence of high concentration of secondary compounds such as polyphenols and polysaccharides in insect tissues, the efficient method for DNA isolation must be optimized by different insect species even to each insect tissue.

The effect of different concentration of β-mercaptoethanol and NaCl on quality of extracted DNA widely has been reported (7, 13, 23). Increasing levels of these variables, along with CTAB, usually results in polyphenols and polysaccharides removal during DNA extraction. In the present study, the DNA extraction buffer containing 2% β- mercaptoethanol and 1M NaCl was used. Our results are in accordance with the results of Kumar Sahu *et al*. that have reported for the DNA extraction protocol from plant tissue, using high concentrations of β-mercaptoethanol and 1.5 M NaCl in extraction buffer (23). Lagisz *et al* have reported simple and high throughput method for DNA extraction from insects, however, in this protocol 10 mg/ml of proteinase K has been used (2). Also, Calderon-Cortes *et al*., have used a simple and rapid method for DNA extraction from Xylophagous insects using CTAB and CTAB-PVP modified method. However, in this case, insect's tissues were powdered in liquid nitrogen (7). Until now, there is not an optimized protocol for efficient DNA extraction from the Green Lacewing species. In conclusion, the work presented here is the first report for a rapid, inexpensive and efficient protocol of high yield geno-

mic DNA extraction from Green Lacewing species as the sample of insects. Extracted DNA with this method is suitable for PCR and PCR-based methods. Also, in current optimized protocol, we have omitted the need of using liquid nitrogen and hazardous or toxic chemicals such as phenol and enzymatic agents like RNase and proteinase K. Our results, derived at laboratory scale, can be used as a basis for further investigation involving DNA extraction from mammalian cells and animal or plant tissues.

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