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A simple, fast, and inexpensive CTAB-PVP-silica based method for genomic DNA isolation from single, small insect larvae and pupae

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ABSTRACT. In this study, we report a modified CTAB-PVP method combined with silicon dioxide (silica) treatment for the extraction of high quality genomic DNA from a single larva or pupa. This method efficiently obtains DNA from small specimens, which is difficult and challenging because of the small amount of starting tissue. Maceration with liquid nitrogen, phenol treatment, and the ethanol precipitation step are eliminated using this methodology. The A_{260}/A_{280} absorbance ratios of the isolated DNA were approximately 1.8, suggesting that the DNA is pure and can be used for further molecular analysis. The quality of the isolated DNA permits molecular applications and represents a fast, cheap, and effective alternative method for laboratories with low budgets.

Key words: Genomic insect DNA extraction; Larva and pupa; CTAB-PVP-silica based method

INTRODUCTION

Insects are the most diverse of all animal groups, but the larval stages are unknown in most species. Research on larvae is important from an ecological and taxonomic point of view. From an ecological viewpoint, the determination of larval stages is indispensable to estimate the functional role of a species in an ecosystem (Klimes and Saska, 2010) and from a taxonomic standpoint, larval morphology provides valuable phylogenic information that cannot be obtained from adult morphology (Beutel and Leschen, 2005).

Molecular markers, such as random amplified polymorphic DNA (RAPD), single sequence repeats (SSR), and amplified fragment length polymorphism (AFLP), have allowed the genetic characterization of different populations of insects (Chen et al., 2008; Gupta and Preet, 2012). Novel DNA barcoding-based methods are available for identifying the species of insect larvae (Dittrich-Schroder et al., 2012). DNA from unidentified larval samples collected from the field is compared with identified adult samples (Vargas et al., 2014). This methodology has allowed determination of previously unknown morphological and ecological traits of many species during their larval stages (Hayashi and Sota, 2010). Isolation of high quality DNA is an essential prerequisite for any PCR-based molecular tool, and the ability to prepare and isolate genomic DNA from several sources is an important step in many molecular techniques.

Homemade insect DNA isolation methods are cheap and very efficient at producing DNA samples, but are usually time-consuming and less efficient in the removal of protein, carbohydrates, and other organic contaminants from samples, thus, causing enzymatic inhibition in subsequent molecular analyses (Hajibabaei et al., 2005; Chen et al., 2010). Commercial kits are generally very fast but very expensive, and generate large quantities of contaminant wastes such as beads, filters, columns, and microcentrifuge tubes.

Recently, several methodologies to isolate genomic DNA from adult insects have been reported (Chen et al., 2008; Gupta and Preet, 2012; Wang and Wang, 2012). Although there are few reports about genomic DNA isolation from larvae and pupae (Dittrich-Schroder et al., 2012), there are fewer reports related to obtaining sufficient quantities of high-quality DNA for molecular work when the larvae or pupae are very small.

In this study, we develop a new method to obtain good quality DNA from larvae and pupae, including single and very small specimens. This method combines CTAB-PVP-extraction and silicon dioxide (silica)-based protocols. Our method is rapid, simple, inexpensive, efficient, and less hazardous to health than other methods described previously.

MATERIAL AND METHODS

Insect material

Larvae and pupae of *Angelabella tecomae* and *Macaria mirthae* were donated by the Laboratorio de Entomología of the Universidad de Tarapacá, Arica, Chile. These samples were preserved in 95% ethanol.

DNA extraction protocol

For DNA isolation, entire larva or pupa were transferred to a 1.5 mL centrifuge tube, ground using a plastic pestle, and immediately mixed with 500 µL lysis buffer (2% CTAB, 100

mM Tris-HCl, 1.4 M NaCl, 1% PVP, 20 mM disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA), 0.2% LiCl. The pH was adjusted to 8.0 before autoclaving. After vortexing and incubating at 65°C for at least 30 min (gently shaken every 10 min), the mixture was centrifuged at 10,000 rpm for 5 min at room temperature (RT). The supernatant was transferred into a new 1.5 mL centrifuge tube and an equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added. Then, the tube was gently flipped several times. After centrifugation for 10 min at 12,000 rpm, the supernatant was transferred into a new 1.5 mL centrifuge tube, followed by the addition of 1 volume of 3 M NaCl and 25 µL silica matrix (1 mg/mL). The mixture was vortexed and incubated for 5 min at RT and then centrifuged at 12,000 rpm for 11 s. The pellet was washed twice with 500 µL washing solution (50% ethanol, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA). DNA was eluted by re-suspending the silica matrix pellet with 50 and 28 µL of distillated deionized H_2O for *M. mirthae* and *A. tecomae*, respectively, and then incubated at 65°C for 5 min and centrifuged at 12,000 rpm for 2 min at RT. Approximately 45 and 25 µL supernatant containing the eluted DNA from *M. mirthae* and *A. tecomae*, respectively, were carefully transferred to new tubes without disturbing the silica particles.

Preparation of silica matrix

The procedure to prepare the size-fractioned silica particles was undertaken according to Li et al. (2010). Silicon dioxide (5 g; S5631, Sigma-Aldrich, Saint Louis, MO, USA) was mixed with 50 mL sterile water in a 50 mL Falcon tube and allowed to settle overnight. The supernatant containing fine silica particles was removed and the pellet was re-suspended in 50 mL sterile water and re-settled overnight. The supernatant was removed and the pellet was re-suspended in 50 mL sterile water.

DNA amplification and sequencing reactions

Polymerase chain reaction (PCR) was carried out using the universal barcoding primers LepF (5'-ATTCAACCAATCATAAAGATATTGG-3') and LepR (5'-TAAACTTCTGGATGT CCAAAAAATCA-3') (Hebert et al., 2004) to yield an approximately 690-bp fragment of the cytochrome oxidase subunit I (COI) region of the mitochondrial DNA. PCR was performed in a final volume of 20 µL. Each reaction contained 1 µL DNA extract, 10 ρmoles of each primer, 2.5 mM of each dNTP, 2 mM $MgCl_2$, 1X PCR buffer (KCl), 1 unit of Taq DNA polymerase (Thermo Scientific) and sterile distilled water. Cycling conditions were: 5 min at 94°C; 35 cycles of 30 s at 94°C; 30 s at 55°C; 30 s at 72°C, and a final elongation step of 2 min at 72°C. PCR blank controls were incorporated. PCR products (3 µL of each) were visualized on 1.5% agarose gels stained with gel-red (Biotium). Reactions containing fragments of the expected size were directly sequenced by a commercial facility (Macrogen, South Korea).

RESULTS

We tested this method with larvae and pupae from *A. tecomae* and *M. mirthae* (Figure 1).

The integrity of the genomic DNA isolated using this method was visualized on 1.2% agarose gels (Figure 2A). DNA samples showed intact/clear bands on agarose gels, suggesting that little or no DNA degradation had occurred during the extraction (Figure 2A). Spectrophotometer analysis showed that the A_{260}/A_{280} ratios of DNA samples ranged from 1.66 to 1.94 (N

= 7) and 1.62 to 1.93 (N = 6) for *M. mirthae* and *A. tecomae*, respectively, suggesting that the DNA fraction was pure and may be used for further analysis. In all samples, the A_{260}/A_{230} ratios were greater than 1.6, suggesting minimum contamination by polysaccharides. The yields were 300 and 156 ng/µL for *M. mirthae* and *A. tecomae*, respectively.

Figure 1. Pupa and larva of *Angelabella tecomae* (left) and pupa of *Macaria mirthae* (right). Scale bar: 1 mm.

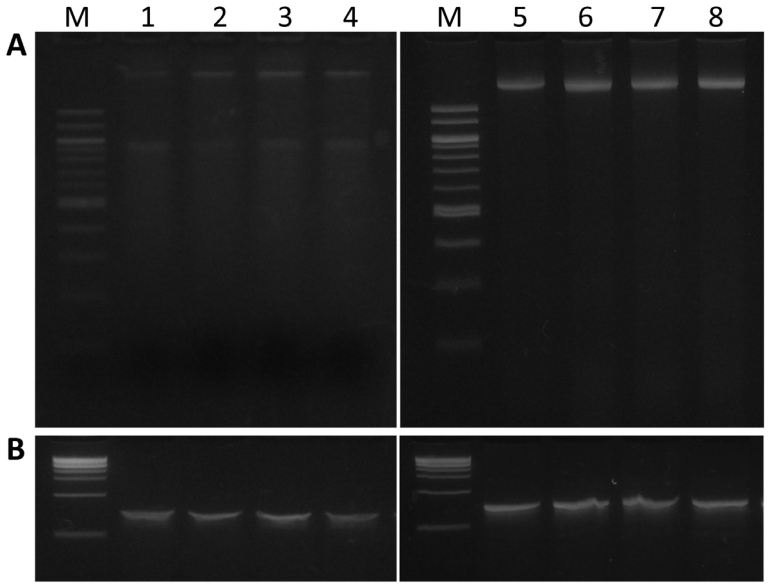

Figure 2. Total genomic DNA analysis (**A**) and amplification of the cytochrome oxidase subunit I gene fragment (**B**) using total genomic DNA extracted from larvae and pupae of *Angelabella tecomae* (1-4) and *Macaria mirthae* (5-8) with the CTAB-PVP-silica method. Samples 1, 2, 5, and 6 correspond to genomic DNA from a single larva and samples 3, 4, 7, and 8 correspond to genomic DNA from a single pupa. *Lane M* = 100-bp (A) or 1-kb DNA ladder (B).

The quality of the genomic DNA isolated using this method and its usefulness for basic molecular analysis was evaluated by PCR. A single fragment of approximately 690 bp of COI was successfully amplified in all samples analyzed (Figure 2B). These PCR products generally yielded sequence read lengths of 690 bp during DNA sequencing analysis (Figure 3), which also reflects the high quality of the DNA template generated by this method.

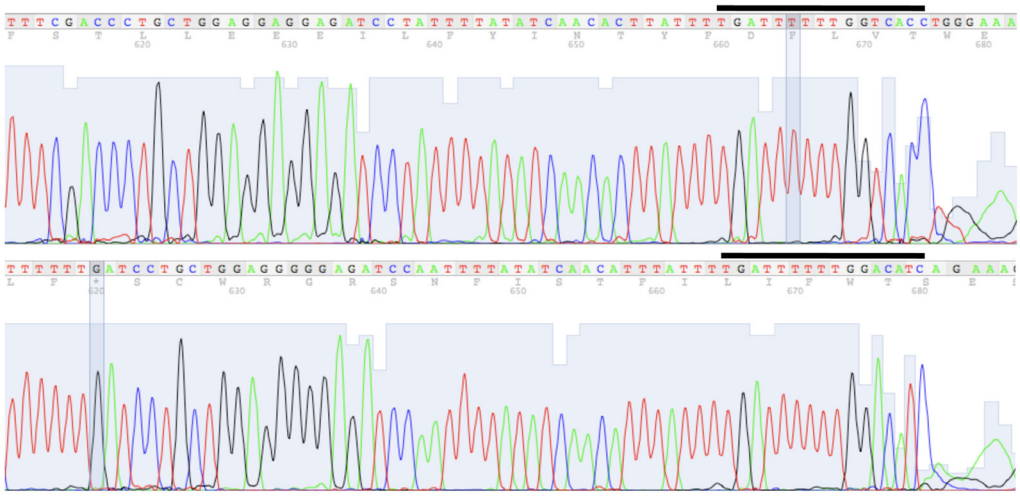

Figure 3. DNA sequencing of the cytochrome oxidase subunit I region in *Macaria mirthae* (upper panel) and *Angelabella tecomae* (lower panel). The 3'-end of each DNA sequencing result is shown with individual nucleotide peaks clearly distinguishable. Fragments of the primer sequence are indicated by a black line.

DISCUSSION

A simple and efficient protocol for genomic DNA extraction is of great scientific value for a wide range of applications. In this study, we developed and evaluated the effectiveness of a simple, fast, and low-cost procedure to successfully extract genomic DNA from larvae and pupae, including small samples. We choose *A. tecomae* because its larvae and pupae are around 1.5 mm in length (Figure 1), and thus, represent a good system to demonstrate the effectiveness of this method. The CTAB-PVP-silica DNA extraction protocol combines the CTAB-PVP method (Atashpaz et al., 2010) and the silica binding procedure (Li et al., 2010). The CTAB-PVP method was previously developed to isolate bacterial genomic DNA and showed excellent results in several genomic DNA extractions of fungi (Huanca-Mamani et al., 2014). This method does not require liquid nitrogen to grind the samples. The presence of phenolic compounds and polysaccharides can co-precipitate with DNA and inhibit enzymatic reactions in subsequent analyses (Lodhi et al., 1994; Calderón-Cortes et al., 2010). In this method, CTAB and PVP were used to remove these contaminants. CTAB separates the polysaccharides from DNA during extraction (Möller et al., 1992; Lodhi et al., 1994; Rouhibakhsh et al., 2008) and PVP forms complex hydrogen bonds with phenols and co-precipitates with cell debris upon cell lysis (John, 1992; Puchooa and Venkatasamy,

2005). The RNA is eliminated from samples through selective precipitation using LiCl present in the lysis buffer. β-mercaptoethanol is not used because it may be toxic to some extent (Rouhibakhsh et al., 2008).

Due to the ability of DNA to bind to silica in the presence of chaotropic salt (Boom et al., 1990), it has been widely used in DNA isolation, being a very efficient, inexpensive and rapid means to isolate DNA from several sources (Carter and Milton, 1993: Hoss and Paabo, 1993; Lakshmi et al., 1999; Dederich et al., 2002; Li and Sheen, 2010).

Amplifications of the COI fragment were obtained after the DNA samples had been stored for at least eleven months (data not shown), indicating that DNA isolated by the CTAB-PVP-silica method is suitable for longer storage periods.

The advantage of this method is its low cost. Maceration with liquid nitrogen, phenol treatment, and the ethanol precipitation step are all eliminated using this methodology. All solutions used in this protocol are made from common and inexpensive chemicals. The original protocol used sodium iodide (NaI) as the chaotropic salt to allow binding of DNA to silica (Li et al., 2010), which is an expensive chemical. We replaced it with NaCl, which produced better results than NaI (Rohland and Hofreiter, 2007) and it is cheaper, so the total cost for 50 reactions is less than US$ 5.

In conclusion, this is the first report that uses silica in an insect DNA extraction protocol. This method is an efficient, inexpensive, and highly reproducible technique to isolate total genomic DNA from tissues of larvae or pupae, obtaining a good yield of high-quality DNA, even from very small specimens for several molecular applications, and represents a viable alternative in laboratories with low budgets.

Conflicts of interest

The authors declare no conflict of interest.

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