

Full Length Research Paper

Rapid isolation of high molecular weight DNA from single dry preserved adult beetle of *Cryptolaemus montrouzieri* for polymerase chain reaction (PCR) amplification

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For studying genetic diversity in populations of predatory coccinellid, *Cryptolaemus montrouzieri* Mulsant (Coccinellidae: Coleoptera), our attempts to isolate high quality DNA from individual adult beetle using several previously reported protocols and even modifications were quite unsuccessful as the insect size was small and was preserved at -20°C as dry specimen. Here we describe a simple, rapid and efficient method of isolating high-quality intact genomic DNA with reduced protein contamination for polymerase chain reaction (PCR) amplification from a single, dry preserved specimen of adult *Cryptolaemus*. The procedure features macerating and mixing the single adult specimen of *Cryptolaemus* with cationic detergent cetyltrimethylammonium bromide (CTAB) in the homogenization buffer, two chloroform-isoamylalcohol extractions and an alcohol precipitation. RNA contamination was eliminated with RNase treatment. The purity of DNA was high since the A_{260}/A_{280} ratio ranged from 1.78 to 1.97. The isolated DNA was used as template for PCR, and the results were evaluated by comparing with different preserved samples.

Key words: Rapid isolation, quality DNA, dry preserved specimens, *Cryptolaemus montrouzieri*.

INTRODUCTION

The added advantage of the molecular marker studies has greatly enhanced the speed and efficiency of insect population studies. To take advantage of these methods, adequate quantity and quality of DNA is required. The isolation of DNA in insects normally does not present any specific problems and any one of a multitude of techniques used for isolation of DNA from other organisms will usually work with insect tissues (Andrew and Gary, 1996). Nevertheless, the major problems usually encountered were small size of the insect,

presence of thick exoskeleton and high quantities of proteins. Further, the chemotypic heterogeneity among species may not permit optimal DNA yields from one isolation protocol, and perhaps even closely related species may require different isolation protocols (Weishing et al., 1995). Usually, it is highly recommended to isolate DNA from fresh tissues but sometimes the samples may be obtained in dry or semi-dry condition. These inevitable conditions led to a development of a protocol for isolating DNA from dry and dead samples as well as live samples of *C. montrouzieri*. This protocol led to the isolation of good quantity of DNA without protein contamination which can be used for PCR amplification and further downstream applications.

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Table 1. Quantification of DNA yields in different treatments.

Sample	Concentration (ng/ μ l)	A ₂₆₀ /A ₂₈₀
Live (T ₁)	1725.0	1.97
Dead (from room temperature) (T ₂)	1258.0	1.86
Stored as dry specimen at -20°C (T ₃)	900.0	1.78
Stored in 70% ethyl alcohol (T ₄)	1025.0	1.78
Stored in absolute ethyl alcohol (T ₅)	784.0	1.92

MATERIALS AND METHODS

Sample preparation

The *C. montrouzieri* samples used were collected from the laboratory established culture at Indian Institute of Horticultural Research, Bangalore. The samples were kept under different conditions viz., live (T₁), dead at room temperature (T₂), dry preserved at -20°C (T₃), preserved in 70% ethyl alcohol (T₄), preserved in absolute ethyl alcohol (T₅). Prior to DNA extraction, the samples were taken in 1.5 ml Eppendorf tubes, washed with sterile distilled water and wiped with tissue paper.

DNA extraction

The samples were crushed thoroughly using a micro pestle. To each sample, 250 μ l of CTAB lysis buffer (2%, w/v) was added and mixed thoroughly. The samples were incubated in water bath at 50°C for 25 min. The samples were left at room temperature for 5 min followed by addition of 250 μ l chloroform: isoamylalcohol (24:1) and the contents were mixed well by slowly inverting the tubes. The contents of each tube were centrifuged at 8000 rpm at 4°C for 15 min and the aqueous phase was collected carefully in to new tubes. The chloroform: isoamylalcohol step was repeated and the supernatant was transferred carefully into a new tube and equal volume of ice-cold isopropanol was added and mixed well before keeping for precipitation at -20°C for 1 h. The samples were centrifuged at 8000 rpm at 4°C for 15 min and the supernatant was discarded. The pellet obtained was washed with 70% ethyl alcohol and centrifuged at 6000 rpm at room temperature for 8 min. The DNA pellet was dried for 1-2 h until the smell of ethanol completely undetectable and resuspended in 25 μ l of molecular grade water. The DNA samples were subjected to overnight RNase treatment (2 μ l con. RNase (10 mg/ ml Fermentas) + 98 μ l molecular grade water). Isolated DNA samples were stored frozen at -20°C.

DNA quantification

Final DNA quantification was done using Nanodrop (ND-1000 Spectrophotometer- V3.2) according to manufacturer's protocols. For quantification of DNA, absorbance was measured at 260 nm. A solution with an OD₂₆₀ of 1 contains 50 μ g of DNA per milliliter (Sambrook et al., 1989). Purity of isolated DNA was checked by obtaining mean of the absorbance ratios (A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀) for proteins and polyphenols/polysaccharide compounds, respectively.

PCR amplification

Each PCR mixture (25 μ l) contained 19.7 μ l of PCR grade water, 0.5 μ l of each primer (0.2 pmol; Bio-serve Pvt. Ltd, India), 0.5 μ l of dNTPs (10 mM; Fermentas), 2.5 μ l (10X) of Taq buffer (Genei), and 0.3 μ l of Taq DNA Polymerase (Genei) and 1 μ l of DNA (25 ng/ μ l).

Amplifications were carried out in Eppendorf master cycler gradient (Germany). The primer pairs ITS F (AGAGGAAGTAAAAGTCGTAACAAG) and ITS R (ATATGCTTAAATTCAGGGGG) were used for the amplification of region including the ITS 1, 5.8 S and ITS 2 (Hurtado et al., 2008). Amplification reactions were performed in master cycler with heated lid. The initial denaturation for 5 min at 94°C was followed by 35 cycles of 35 s at 94°C, 30 s at 43°C, 35 s at 72°C and a final extension of 10 min at 72°C. The PCR products were gel electrophoresed on 1.5% agarose gel.

Validation

The validation of developed DNA isolation protocol was tried with archival (10 years old) dry preserved single specimen of fruit fly, *Bactrocera dorsalis*. The adult fruit fly specimens of *B. dorsalis* preserved at room temperature as museum specimens were used for this purpose and PCR was carried out with MCOI primers.

RESULTS

The protocol described was found to be suitable for the extraction of genomic DNA from single adult beetle of *C. montrouzieri* samples and achieved high amount of quality DNA from different samples without protein and RNA contamination (Figure 1A). The DNA yield was approximately 1725, 1258, 900, 1025 and 784 ng/ μ l with T₁, T₂, T₃, T₄ and T₅, respectively (Table 1). The DNA was successfully used for PCR amplification to amplify specific fragments of ITS (1500 bp) (Figure 1B).

The amplicons were used to generate sequence data for the ITS region (data not shown). The DNA extraction protocol described here was also used to generate RAPDs in several samples of different isofemale lines/geographical strains. This indicated that the DNA samples were free of any PCR inhibitors. Further, no inhibition of Taq DNA polymerase activity was observed.

The validation of the developed protocol with archival specimens (decade old museum specimens) of *B. dorsalis* also yielded PCR quality DNA which was amplified using MCOI primers (Figure 1C) which otherwise did not yield any quality DNA with existing protocols.

DISCUSSION

As intact DNA isolation was main aim of modification of DNA extraction protocol, DNA extracted by present

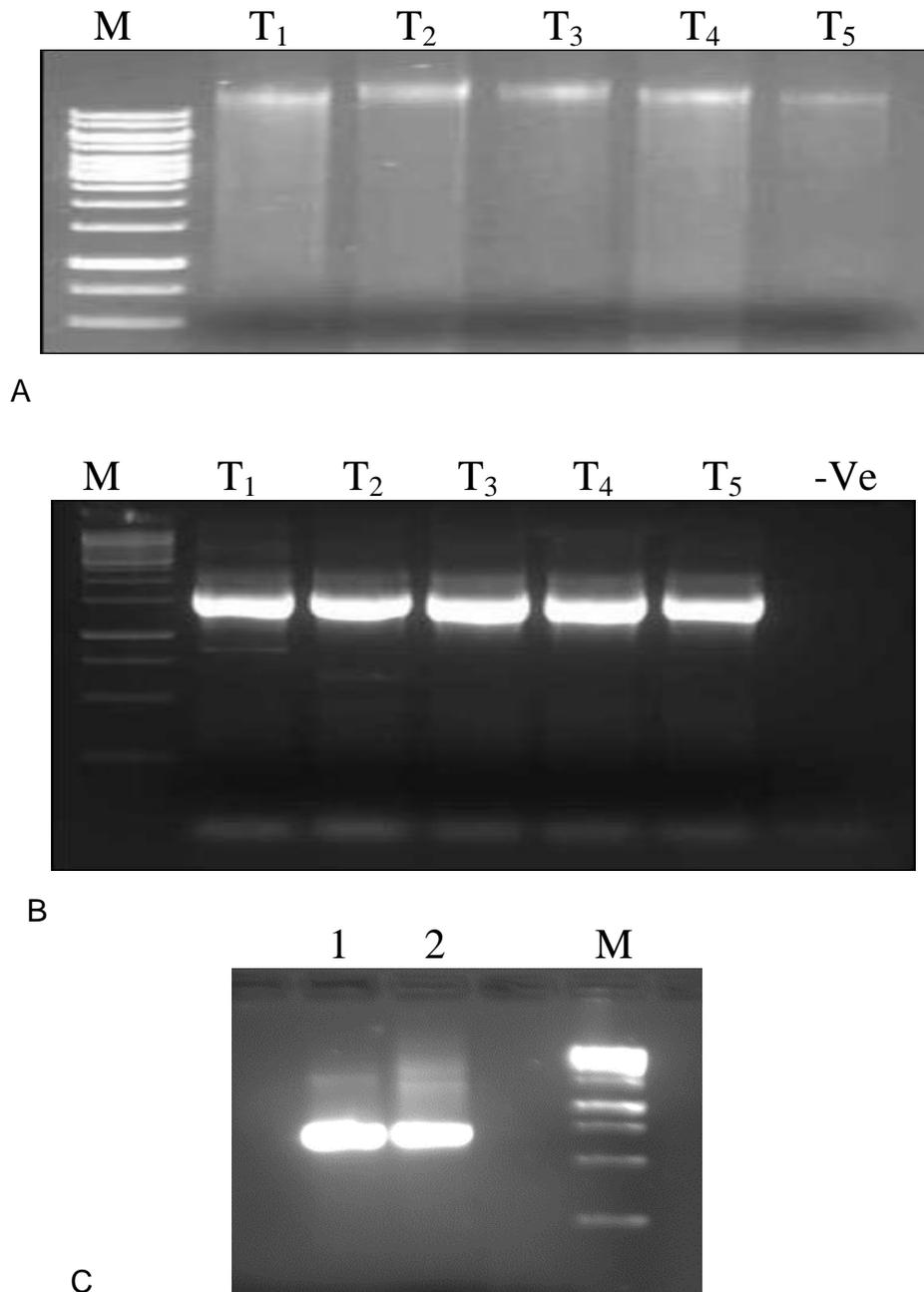


Figure 1. A: Genomic DNA of *Cryptolaemus* using present method, M- marker (1 kb Fermentos); T₁: live; T₂: dead at room temperature; T₃: stored as dry specimen at -20°C; T₄: stored in 70% ethyl alcohol; T₅: stored in absolute alcohol. B, PCR amplification of ITS region (1500 bp) from different *Cryptolaemus* samples using the DNA extracted following the present method; M- Marker (1 kb-Fermentos); T₁; T₂; T₃; T₄; T₅; -Ve, negative control (without template); C:1: PCR amplified DNA (1.5 kb) of fresh samples of *B. dorsalis* using MCOI primer, 2: PCR amplified DNA (1.5 kb) of archival samples of *B. dorsalis* using MCOI primer; M, marker.

method was proved to be more compatible with polymerase chain reaction (PCR)-based technologies and other molecular studies. It has been reported previously that shearing of DNA during extraction can directly or indirectly interfere with the enzymatic reactions

(Weishing et al., 1995) during different molecular studies, like, PCR, restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) etc. However, in the present study, inhibition of Taq DNA polymerase was not noticed. Usually, the insect DNA

isolation protocols use proteinase K to digest all proteinaceous matter (José et al., 2004). However, our protocol eliminates the use of proteinase K as well as liquid nitrogen. Further, it skips overnight incubation for precipitation of DNA, which is usually practiced in case of animal tissues making DNA extraction rapid. The DNA is precipitated using isopropanol and further, the addition of β -mercaptoethanol is helpful in removing poly phenols. It has been reported that high level of β -mercaptoethanol successfully removes the polyphenols (Suman et al., 1999). Usually the insect samples contain a large amount of protein that hampers DNA extractions. In the present method, chloroform: isoamylalcohol (24:1) were used to remove proteins from the samples. Isolation of DNA from archival specimens at times is crucial and the present method was found to be successful to get PCR quality DNA from decade old dry preserved single museum specimens of *B. dorsalis* which otherwise was not possible using existing methods. This method will probably hold good for still older senescent specimens.

DNA yield is of paramount importance when target tissue for obtaining DNA is limited because of sample uniqueness or size of insect. The protocol described here is simple and facilitates rapid isolation of PCR amplifiable genomic DNA (1200 ng/ μ l lasting for 2500 PCR reactions) from single beetle (weighing ~400 mg) and particularly suitable for single, dried specimens of taxonomic value.

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