Pakistan J. Zool., vol. 44(2), pp. 421-426, 2012.

Comparison of Methods for DNA Extraction From a Single Chironomid for PCR Analysis

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Abstract: Genomic DNA of single chironomid midges was extracted using the Cetyl-Trimethyl Ammonium Bromide (CTAB) method, the Proteinase K method and the Chelex-100 method, respectively. The results suggested that all three methods are effective in extraction of genomic DNA of small insects. But comparatively, the quality of DNA sample isolated using the CTAB method was better than those extracted using the other two methods, if the specimens were stored in 75% ethanol for a long time. However, if the specimens were fresh or stored in 95% ethanol, the Chelex-100 and Proteinase K methods, using less chemicals hazardous to health, are more rapid than the CTAB method.

Keywords: Chironomidae, DNA-extraction, PCR amplification, CTAB, proteinase K, Chelex-100.

INTRODUCTION

With molecular biology technology developing dramatically in width and depth, phylogenetic investigations on insect have relied increasingly on molecular biological techniques using PCR-based manipulations of informative DNA markers for detecting the genetic diversity. In order to apply these analytical techniques, a key step is to obtain acceptable quality and quantity of genomic DNA from small specimens for PCR amplification. Especially in the investigation of phylogeny, the process of the DNA extraction should not affect the information of morphology. But many samples are very small, such as the adult male of the genus *Corynoneura* usually less than 1mm (Cranston *et al*., 1989). The primary problem to be solved is how to obtain high-quality DNA samples from single and small specimen.

Family Chironomidae, widely distributed insects in freshwater, belong to true flies (Order Diptera) and were erected by Macquart in 1838. There were estimated to be as many as 15000 species in the world-wide (Cranston, 1995). Over the past ten years, the molecular information has been widely used in the investigation of the phylogeny for many insect groups. A few molecular biology researches have been carried out in family Chironomidae. Ekrem and Willassen (2004) investigated the Tanytarsini relationships using mitochondrial COII gene sequences. In the study of Willassen (2005), 368 bp DNA sequences of COII were used to associate two single males in the material with conspecific females. Carew *et al*. (2005) showed how DNA methods helped in separating three species of Australian *Cladopelma* midge by using COI polymerase chain reaction-restriction fragment-length polymorphism (RFLP) marker. Ekrem *et al*. (2007) examined the possibility of utilizing partial COI gene sequences as barcodes to identify chironomids. In this paper, the methods of CTAB, Proteinase K and Chelex-100 were used in the genomic DNA isolation of chironomids. This is the first report on the comparison of DNA extraction methods from single chironomid for PCR analysis.

MATERIALS AND METHODS

Materials

Chironomid specimens used in this study belongs 2 subfamilies (Orthocladiinae and Chironominae). The fresh samples were placed in 95% and 75% ethanol respectively, and then stored at 4°C. Details of specimen setups are shown in Table I.

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0030-9923/2012/0002-0421 $ 8.00/0

Table I.- Specimens used in this study.

No.	Specimen	Type	The Time	Stored methods
1.	*Bryophaenocladius cuneiformis*	Male	2008	Stored in 95% ethanol
2	*Chironomus samoensis* group	Male	2008	Stored in 95% ethanol
3.	*Cricotopu bicinctus*	Male	2000	Stored in 75% ethanol
4.	*Smitta atterima*	Male	2000	Stored in 75% ethanol

Tissue pretreatment

To analyze the morphological characteristics of the samples, one side of wing, legs, and hypopygium were dissected from single chironomid and mounted on the slide with the conventional method. The remains of body were placed in 500 µL TE (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) buffer or 0.9% sodium chloride (NaCl) for 2 hours. If the samples had been stored in 75% ethanol, they were placed in buffer over night at 4°C, then discarded the flow-through, then added 50 µL extraction buffer, grinded up the samples using a tip until the sample was completely homogenized, then washed the tip with 100 µL extraction buffer. The tip was burned in alcohol and closed at the point in order to reduce attrition of samples in grinding step. Grinding with a tip was also effective in the case of pestle in this method.

DNA extraction

We designed three protocols for DNA extraction from a single chironomid.

Cetyl-trimethyl ammonium bromide (CTAB) protocol

The procedure of DNA extraction followed the method of Bossier *et al*. (2004). 500 µL 2×CTAB buffer (2% CTAB, 1.4 mol/L NaCl, 20 mmol/L EDTA, 100 mmol/L Tris-HCl) and 16 µL Proteinase K (10 mg/ml) were added to the homogenate, then incubated at 56°C for 4-6 hours, and then extracted with an equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1). DNA was precipitated by adding 2-2.5 volumes of 100% cold ethanol. After 2-2.5 hours incubation at -20°C, DNA was washed with 70% ethanol. The pellet was resuspended and dissolved in 25 µL of TE buffer, stored in the refrigerator for subsequnent PCR amplication.

The proteinase K protocol

The DNA was extracted according to the method of Wen and He (2003). 30 µL STE buffer (0.1mol/L NaCl, 1 mmol/L EDTA pH 8.0, 10 mmol/L Tris-HCl, pH 8.0) and 2 µL Proteinase K (10mg/ml) were added to the homogenate. To reduce the loss, the grinding tip must be washed by 30 µL STE buffer again. The samples were incubated at 56°C for 2 hours, and then incubated at 95°C for 45 seconds, centrifuged at 8000-10000 rpm for 30 seconds. The supernatant being used as amplification template for PCR amplification was stored in the refrigerator.

Chelex-100 protocol

DNA extraction procedure was performed according to the method of OuYang *et al*. (2009) modified. 100 µL Chelex-100 (6%) and 8 µL proteinase K (10 mg/ml) were added to the homogenate, then vortexed in chelex slurry for 10-15 seconds, centrifuged samples at high speed for 10-15 seconds. The samples were incubated at 65°C for 1 hour and then boiled for 10 minutes, vortexed again for 10-15 seconds, centrifuged at 14000 rpm for 20 minutes. The supernatant, used as amplification template in PCR reaction, was stored in the refrigerator. In this protocol, vortexing thoroughly is a key step.

DNA quantification and visualization

The sampled DNA was diluted 20-25 times with ddH_2O and quantified by taking the optical density (OD) at 260 and 280 with a spectrophotometer. 5µL DNA was run at 5 V/cm for 40 minutes on a 0.8% agarose gel and then photographed under UV illumination in the GDS-8000 gel image analysis system (SYNGENE, UK).

PCR amplification

PCR amplification was carried out in a PTC-200 (MJ) machine in 50 µL reaction volumes. PCR conditions and primers used are shown in Table II. 5 µL PCR products were run at 5 V/cm for 40 minutes on a 1.5% agarose gel and then photographed under UV illumination in the GDS-8000 gel image

Table II.- PCR amplification conditions and primers used in this study.

Gene	Primer	Sepuense(5'-3')	Denaturation	Annealing	Extension	Reference
CoxII	C2-J-3400 TK-N-3785	ATTGGACATCAATGATATTGA GTTTAAGAGACCAGTACTTG	94°C	45°C-50°C	72°C	Willassen (2005)
28SrDNA D3-D5	485 689	GACCCGTCTTGAAACACGA ACACACTCCTTAGCGGA	95°C	50°C	72°C	Friedrich and Tautz (1997)

analysis system (SYNGENE, UK).

RESULTS

CTAB method was efficient in the genomic DNA extraction from single chironomid (Fig. 1). The DNA extracted by proteinase K and Chelex-100 protocols was too low to be detected on gel.

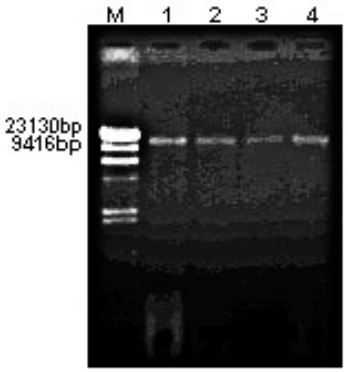

Fig. 1. Genomic DNA extracted using CTAB method on 0.8% agarose gel.
M Lane, λDNA/HindIII+EcoRI molecular weight marker; 1 Lane, *Bryophaenocladius cuneiformis*; 2 Lane, *Chironomus samoensis* group; 3 Lane,: *Cricotopus bicinctus*; 4 Lane: *Smitta atterima*.

Table III shows the total time consumed to extract DNA by the three methods.

The OD 260/280 value of DNA isolated using the CTAB method is 1.83±0.03, exhibiting the best quality and highest purity. They yield was 35-42 ng/µg as against very negligible for the other two methods.

Two genes of the D3-D5 variable region of 28S ribosomal RNA (28S rRNA D3-D5) and cytochrome oxidase subunit 2 (CoxII) were all amplified successfully using the DNA extracted by CTAB, Proteinase K and Chelex-100 methods. The PCR of these two genes showed a single band of 778 bp and 480 bp, respectively (Figs. 2, 3).

Table III.- The time consumed in three different extraction methods

Extraction method	Total time (h)[1]	Total time (h)[2]
CTAB	22.0-24.5	10.0-12.5
PK	16.5	4.5
Chelex-100	16.0	4.0

1, the samples stored in 75% ethanol; 2, the samples stored in 95% ethanol.

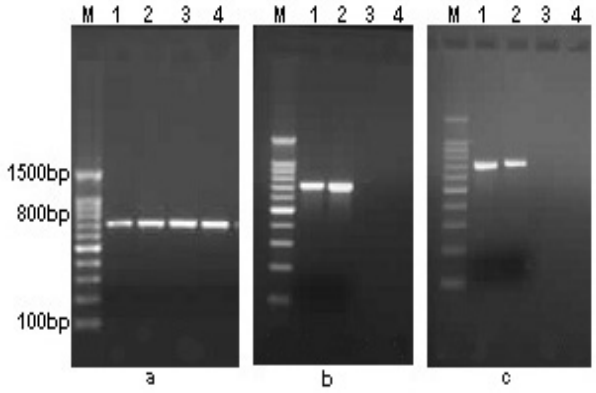

Fig. 2. PCR results for partial gene of 28S rRNA D3-D5 segments amplified using different DNA extraction methods of chironomids. a, the method of CTAB; b, the method of Proteinase K; c, the method of Chelex-100. M Lane, marker (100 bp DNA ladder □); 1 Lane, *Bryophaenocladius cuneiformis*; 2 Lane, *Chironomus samoensis* group; 3 Lane, *Cricotopus bicinctus;* 4 Lane, *Smitta atterima.* The negative results for no DNA controls are not included in the figure.

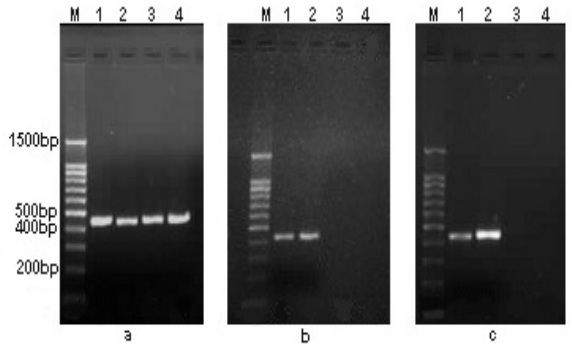

Fig. 3. PCR results for partial gene of CoxII amplified using different DNA extraction methods of chironomids. a, the method of CTAB; b, the method of Proteinase K; c, the method of Chelex-100. M Line: marker (100 bp DNA ladder □); 1 Lane, *Bryophaenocladius cuneiformis*; 2 Lane, *Chironomus samoensis* group*;* 3 Lane, *Cricotopus bicinctus;* 4 Lane, *Smitta atterima.* The negative results for no DNA controls are not included in the figure.

DISCUSSION

Storage methods

Establishing proper sample-storage methods is important for DNA extraction. Now some storage protocols were reported, such as frozen, dried, and alcohol-fixed samples. Piao *et al*. (2002) reported that dried samples stored for a long time failed to amplify successfully, because the genomic DNA was severely degraded and the fragment integrity was violated. Zhang *et al*. (2002) found that oven-dried specimens and those preserved in alcohol were better than naturally dried specimens in DNA extraction from seven insect species, because the naturally dried specimens were more easily attacked by mildew and DNA extracted from these specimens was likely to degrade when the relative humidity was high in the surroundings. Leutbecher (2000) failed to employ random primers for RAPD-PCR using dried and 70% ethanol fixed rotifers. The loss of DNA in ethanol-fixed material may have resulted from damage to the nuclear membrane which allows the DNA to be exposed to cytosolic DNases.

In this study, the samples were stored in 75% and 95% ethanol, respectively. Using the methods of CTAB, Proteinase K and Chelex-100, DNA extracted from samples stored in 95% ethanol all showed a clear band on gel, while only DNA could be amplified successfully in CTAB methods when the samples were stored in 75% ethanol for a long time. Low DNA yield and failed PCR amplifications in Proteinase K and Chelex methods could be due to the presence of impurities such as terpenes and polysaccharides (Shepherd *et al*., 2002). Establishment of effective sample storage and DNA extraction methods have been helpful in effectively synchronizing traditional taxonomy research and molecular phylogeny research of small insects.

Pretreatment method

Pretreatment of samples is an important step in DNA extraction. In this study, it was necessary to immerse samples stored in 75% ethanol in TE buffer for 12-16 hours, because the ethanol remained could affect the subsequent DNA extraction process directly. The key step to obtain high-quality DNA is grinding method of tissue. In previous studies, tissues always were done by using glass-bead grinder or grinding in liquid nitrogen (Jinneman *et al*., 1998; Hnida and Duszynski, 1999). This method is not suitable for small species of chironomids. The smaller the species more the loss is from the above procedure, which will reduce the eventual DNA yield. In this study, a tip suitable for the individual small insect was used to grind the tissues.

Extracted DNA

The ultimate goal of DNA extraction is to obtain DNA with high molecular weight and without impurities. The disruption of cellular and nuclear membranes is the first necessary step to obtain purified DNA. The detergents (Surfactants), such as the sodium dodecyl sulfate (SDS) and CTAB, were adopted to accomplish disruption. In the past, CTAB was always used in plant and fungal DNA extraction (Jose and Usha, 2000; Zhao *et al*., 2001; Mo and Rinkevich, 2001; Sharma *et al*., 2003;), and now this protocol is often used in insects and crustaceans for DNA extraction (Bossier *et al*., 2004; Shi *et al*., 2005; Herborg *et al*., 2007). CTAB could remove possible polysaccharides that might be combined with DNA. Zhao *et al*. (2001) showed that the CTAB incubation step could improve the DNA quality, especially for the plastid DNA and for

a long PCR reaction. The Chelex extraction method is frequently used to release DNA from a low numbers of cells by a boiling treatment, and at the same time protecting the DNA from the boiling effects with resin beads (Walsh *et al*., 1991). In this paper, comparatively, CTAB method provided a higher purity of DNA sample using both 75% and 95% ethanol stored chironomids, and the Proteinase K and Chelex-100 methods were rapid and easy to perform while providing small-scale DNA templates suitable for PCR amplification using 95% ethanol stored chironomids. Moreover, these two methods consumed less time as compared to CTAB method since the usual step of phenol-chloroform was not used.

In conclusions, three protocols were all suitable for the DNA extraction of small chironomids. CTAB protocol could extract high quality DNA from single chironomid stored in 75% ethanol without consuming any expensive reagents, while the samples were fresh or stored in 95% ethanol, the Proteinase K and Chelex-100 methods using less chemicals hazardous to health were the best selections in DNA extraction.

ACKNOWLEDGEMENTS

Financial support received from the National Natural Science Foundation of China (NSFC, 30770249, 308703291, J0630963) and Fauna of China (2006FY120100) are gratefully acknowledged.

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(Received 9 May 2011, revised 23 August 2011)