

Journal of Coastal Life Medicine

journal homepage: www.jclmm.com



Original article doi: 10.12980/jclm.4.2016J6-88

©2016 by the Journal of Coastal Life Medicine. All rights reserved.

A simple and cost-effective protocol for extraction of genomic DNA from ethanol preserved black flies (Simuliidae: Diptera)

Neelamegam Rameshkumar^{1*}, Sankarappan Anbalagan², Nagarajan Kayalvizhi³, Rasiravuthanahalli Kaveriyappan Govindarajan³, Vimalanathan Arun Prasanna¹, Muthukalingan Krishnan¹

¹Department of Environmental Biotechnology, Bharathidasan University, Tiruchirappalli 620024, Tamil Nadu, India

²Department of Zoology, Sethupathy Government Arts College, Ramanathapuram 623502, Tamil Nadu, India

³Department of Zoology, Periyar University, Salem 636001, Tamil Nadu, India

ARTICLE INFO

Article history:

Received 5 May 2016

Received in revised form 29 Jun, 2nd

revised form 30 Jun, 3rd revised form

13 Jul 2016

Accepted 10 Aug 2016

Available online 7 Sep 2016

Keywords:

Black flies

Ethanol preservation

DNA extraction

PCR Amplification

ABSTRACT

Objective: To investigate the efficacy of extraction methodology for the proposed DNA from the ethanol preserved black flies (Simuliidae: Diptera).

Methods: This study addressed a simple and effective protocol for extraction of DNA from black flies stored in the ethanol. The sizes of larval and adult black flies ranged from 1.5 to 2.5 mm and 3 to 7 mm, respectively. To demonstrate the efficacy of the proposed methodology, the DNA was extracted from the ethanol preserved sample of black flies using the commercial kits. The extracted DNA was further validated in the PCR amplification using internal transcribed spacer-1 rDNA and cytochrome oxidase subunit II rDNA target primers.

Results: Interestingly, a minor modification in the proposed methodology yielded a good DNA concentration in comparison with the commercial kits. The extracted DNA sample using the proposed methodology was successfully validated in the PCR amplification using internal transcribed spacer-1 rDNA and cytochrome oxidase subunit II rDNA genes.

Conclusions: The proposed DNA extraction procedure yielded good concentration of DNA from the ethanol preserved black flies. The added advantage is that the procedure is suitable for a range of insect species preserved in the ethanol obtained from the various field conditions.

1. Introduction

Black flies are one of the medically important insects belonging to the family of Simuliidae (order: Diptera). They are important as pests of humans and animals and also act as a vector for filarial nematode of the genus *Onchocerca* to humans and cattle. This nematode causes onchocerciasis or river blindness transmitted by many species of female adult black flies[1]. In addition, black flies can cause a 'black fly fever' with symptoms like headache, fever, nausea, dermatitis and allergic asthma. Larvae of black fly are one

of the major components of the benthos in running waters and they play a vital role in stream ecosystem as a prey for predators and stand as a trophic link[2]. According to Adler and Crosskey[3], a total of 2177 of species belonging to 26 genera of Simuliidae family are distributed in the world. Many species are misidentified because of the extraordinary similarity between species. The young taxonomists have been facing numerous problems in describing the species of black fly. They are mainly concentrating on the morphological features. The sizes of larval and adult black flies ranges from 1.5 to 2.5 mm and 3 to 7 mm, respectively. Since the breeding habitat of black fly is in running waters which flow through forests and urban areas, they depend mainly with water flow, temperature and season[4]. Due to this condition, taxonomists should take more efforts for sampling and rearing of black fly. However, the molecular methods employing in black flies for the description of species would be helpful for identifying and understanding the knowledge of their geographical distribution. The difficulties arise to bring the live black flies from the field to laboratory, since it

*Corresponding author: Neelamegam Rameshkumar, Insect Molecular Biology Laboratory, Department of Environmental Biotechnology, School of Environmental Sciences, Bharathidasan University, Tiruchirappalli 620024, Tamil Nadu, India.

Tel: +91 9566704854

Fax: +91 431 2407045

E-mail: neelamegamramesh@gmail.com

Foundation Project: Supported by Science and Engineering Research Board, Department of Science and Technology – Fast Track Young Scientists Project, Government of India (Ref. No. YSS/2014/000240/Dt. 06.11.2015).

The journal implements double-blind peer review practiced by specially invited international editorial board members.

needs preservation in the field. Ecologists are widely using ethanol for preserving specimens. DNA extraction is the first step in many molecular biology protocols. A variety of methods have been employed to extract insect genomic DNA, and many commercial kits are available[5]. Several studies have emphasized their efficiency, cost, and side effects such as DNA degradation during extraction[6].

Nowadays, several scientific companies offer commercial kits with a lot of advanced technology to accelerate the process of DNA isolation, as an alternative approach to the conventional methods. However, the kits are problematic in their use of toxic chemicals, and a large number of steps, solution and buffer composition of them are not apparent to the researchers[7]. However, limited studies have been focusing on the effect of ethanol preserved samples and its inefficiency in the PCR amplification[8]. Here, we have proposed a simple, efficient procedure that can be employed for the black fly ethanol preserved samples. Our goal was to isolate DNA from ethanol preserved black flies by an easy and effective method and further to evaluate efficacy of the DNA in the PCR amplification.

2. Materials and methods

2.1. Sample collection

The larvae of *Simulium gravelyi*, *Simulium palniense* (*S. palniense*) and *Simulium gurneyae* were collected from the streams of Palani Hills, Southern India. They were collected from the stream substrates (bedrock, boulders, leaf litter, woody debris, etc.) with the help of fine brush and forceps. The adults were collected on riparian vegetation along the stream bank by a sweep net. The collected samples were stored in the ethanol for 1 year. Specimens were preserved in 95%–100% ethyl alcohol and brought to laboratory for molecular analysis.

2.2. Genomic DNA extraction from ethanol preserved black flies

The DNA was extracted from one-year-old ethanol preserved specimens by the modified protocol of Ausubel *et al.*[9]. In addition, three commercial DNA extraction kits were used for comparative assessment, which were QIAamp genomic DNA isolation kit (Qiagen GmbH, Hilden, Germany), HiPurATM insect DNA purification kit (Hi-Media, Mumbai, India) and DNA extraction buffer (GeNei, Bangalore, India).

2.3. Proposed methodology for extraction of DNA from ethanol preserved black flies

One hundred milligrams of black fly larvae were weighed from the three species in 1.5 mL of sterile centrifuge tubes. The tubes were incubated at 37 °C for 60 min in an incubator for evaporation of ethanol content from the specimen. After incubation, they were homogenized in a sterile micropestle with 200 µL of cell lysis buffer

containing 1 mol/L Tris, 4 mol/L NaCl, 10% sodium dodecyl sulfonate, 0.5% ethylene diamine tetraacetic acid and RNase (0.1 mg/mL). About 20 µL of lysozyme (50 mg/mL) was added to them. The homogenized aliquots were incubated at 70 °C for 30–60 min. Then 100 µL of phenol: chloroform: isoamyl alcohol in the ratio of 25:24:1 was added. They were eddied well and centrifuged at 13000 r/min for 10 min at room temperature. The supernatant was collected and transferred to a fresh tube, where 200 µL of ice-cold ethanol was added. Subsequently, they were kept at 20 °C for 30 min. After incubation, the precipitated DNA was centrifuged followed ethanol wash at different concentrations (100%, 90% and 80%), and finally it was dissolved in 50 µL of TE buffer (10 mmol/L Tris-HCl and 1 mmol/L ethylene diamine tetraacetic acid, pH 8.0). DNA was resolved in 1% agarose gel electrophoresis by staining with ethidium bromide.

2.4. PCR amplification using internal transcribed spacer-1 (ITS-1) rDNA and cytochrome oxidase subunit II (COII) rDNA genes

The PCR reaction (20 µL final volume) was carried out using the following components: 1 µL of DNA extract, 10 µL of master mix (GenetBio Corporation, Daejeon, South Korea), 2 µL of each primer (10 picomol) and 7 µL of sterile water. ITS-1 rDNA and COII rDNA genes were taken for studying genetic diversity of black flies[10]. The ITS-1 primer targeting the region: F- 5'-TGGTGTCATGGCCGTTCTTAG-3' and R- 5'-GTCGATGTTTCATGTGTCCTGC-3' and the COII primer targeting region: F- 5'-ATTATGGCAGATTAGTGCA-3' and R- 5'-GTTTAAGAGACCAGTACTTG-3'. The PCR amplification was performed in an ABI Thermocycler (Germany) using the following reactions: (94 °C for 5 min; 40 cycles of 94 °C for 30 s; 52 °C for 1 min; 72 °C for 1 min; 72 °C for 10 min). PCR products (20 µL) were resolved by electrophoresis in 1% agarose gels stained with ethidium bromide. For all analysis, a negative control (sterile water) was included. The amplified products were sequenced and checked with NCBI-Nucleotide BLAST for the confirmation.

3. Results

The DNA extracted from one-year-old ethanol preserved samples was visualized in 1% agarose gel and it showed that all samples produced very low yields of DNA (Figure 1). But modified protocol produced plausible amount of DNA when compared with commercial kits. Since the usage of lysozyme in manual method might possibly enhance the yield of DNA. We observed variations in the yield of DNA among the three black fly species, which might reflect the differences in the level of tissue disruption (Figure 1).

The isolated DNA from commercial kits and manual method were successfully amplified with ITS-1 and COII primers for three black fly species. The PCR products were loaded in 1% agarose gel, and simultaneously the negative control samples were also loaded. The

PCR products from the DNA isolated by modified protocol showed bands with good quality for all the species and their products sizes ranged between 500 and 1000 bp (Figure 2). Comparatively, the low yield or no PCR amplicons was obtained from DNA extracted by commercial kits. The negative control showed no amplification, indicating that there was no significant DNA cross-contamination. The gene sequences of ITS-1 and COII from commercial kits and manual methods for three black fly species were checked. The gene sequences of ITS-1 and COII were amplified through modified protocol which made it readable and gave no repeating errors, whereas the amplicon from DNA of commercial kit provided

repeating errors and less product sizes (< 500 bp). It was due to cross-contamination, less amplification and meager amount of DNA presenting in the sample.

The above results confirmed that the DNA concentration and purity obtained by our new method were highly suitable for molecular biology applications. Apart from the quality of the DNA, our present method was rapid and used less toxic chemicals with fewer steps, which in consequence, should be suitable for molecular biology applications. The analyses provided consistent evidence that DNA extracted by this methodology was sufficient for several PCR amplifications. Therefore, this method was particularly suitable for low-budget research projects

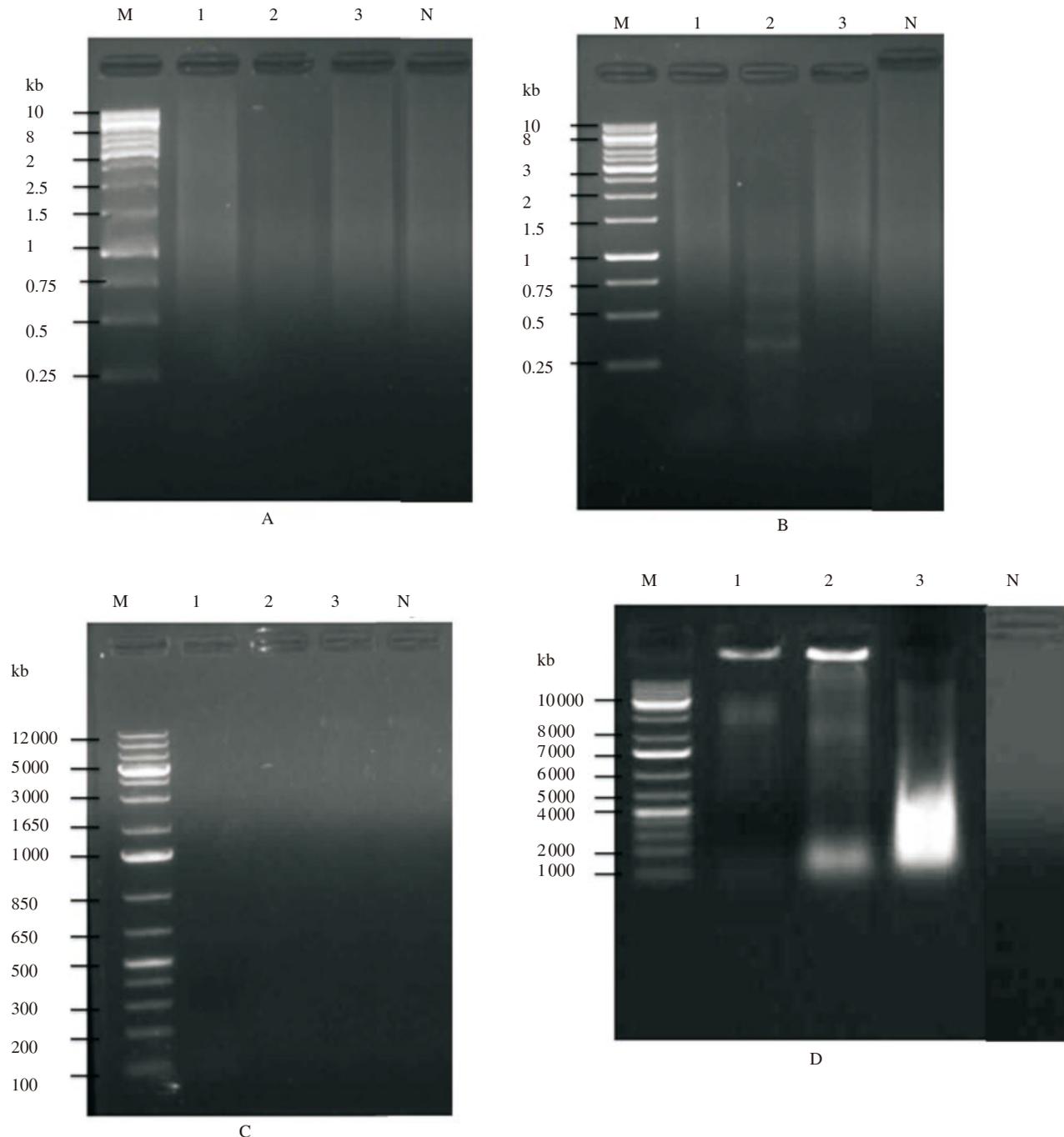


Figure 1. Genomic DNA isolated for three black fly species by four methods.

A: QLAamp DNA isolation buffer; B: HiPurA buffer; C: GeNei DNA extraction buffer; D: Modified manual protocol; M: Marker (1 kb); 1: *Simulium gravelyi*; 2: *S. palniense*; 3: *Simulium gurneyae*.

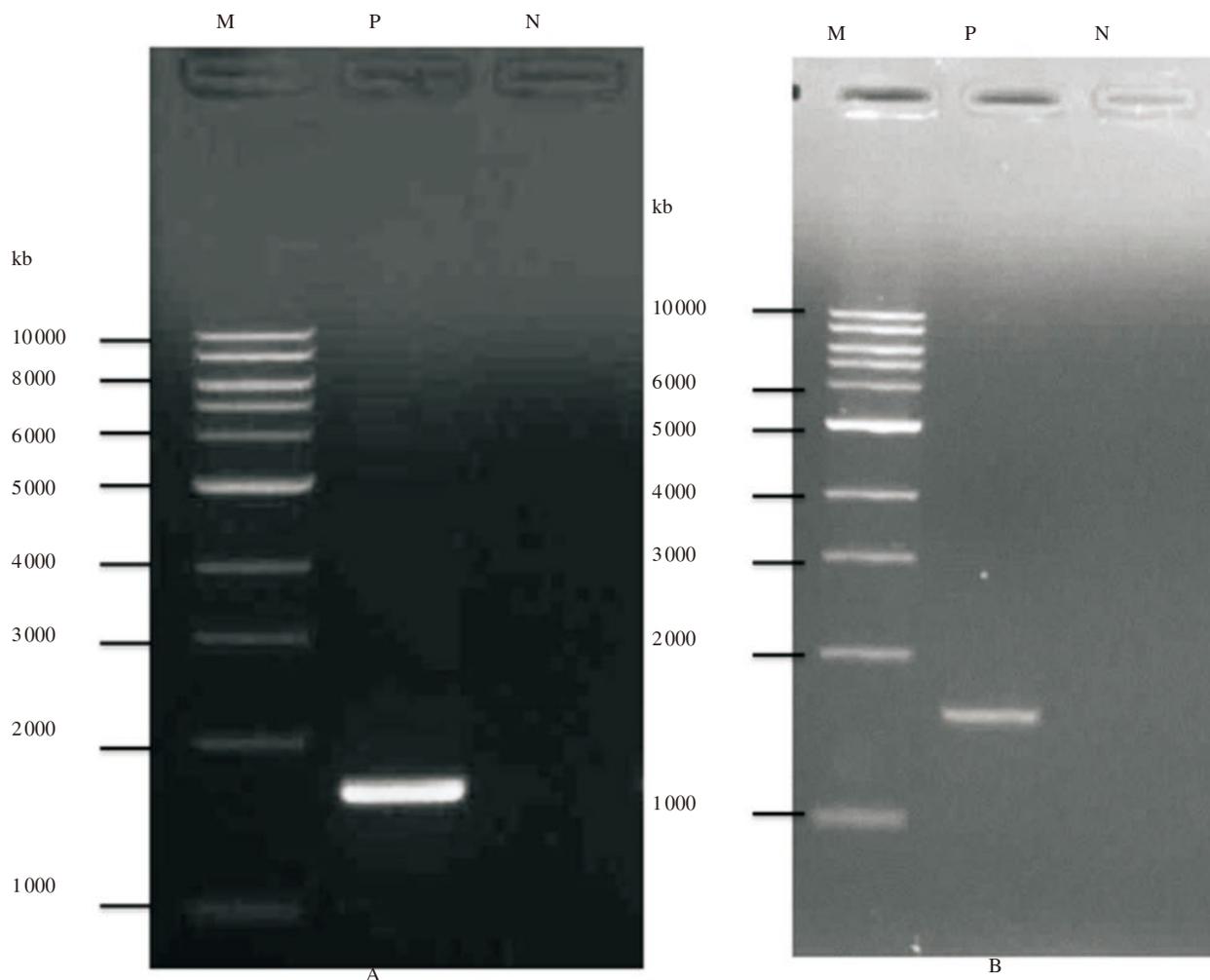


Figure 2. PCR amplification of ITS-1 and COII regions for *S. palniense* species.

A: ITS-1 amplification; B: COII amplification; M: Marker (1 kb); P: Positive reaction; N: Negative control.

and for laboratories with only basic equipment at present. The study concluded that the modified protocol offered a swift DNA extraction from black fly larvae preserved in ethanol within 2 h. It did not need any expensive reagents and equipments during the extraction of high quality DNA, which was suitable for molecular analysis.

4. Discussion

Vector-borne diseases are one-sixth of the infirmity in the world[11,12]. Approximately, one billion people are infected due to vector-borne diseases[13]. Of which, 17.6 million cases are significantly affected by onchocerciasis[11]. Although, onchocerciasis disease or river blindness is carried by adult female black flies. However, no case on onchocerciasis has yet been reported[2]. Nevertheless, molecular taxonomy of black flies from India is needed to determine their phylogenetic level and vector potential.

To identify insect, different molecular techniques are used for research on their role. For each molecular technique, high quality DNA is required, which is extracted from the insects by different techniques. The selection of DNA extraction technique is depended upon specimen under study, time required for extraction and economical usage of technique due to reagents and equipment used

for extraction and most importantly extracted DNA quality[11]. In particular, insects collected from the field condition were stored in ethanol for years and examined in laboratory. Although, the storage conditions preserve the sample intact, it is proved that ethanol or other preservatives have some adverse effects on the samples. Hence, there is a need for a simple and standard protocol for the extraction of genomic DNA. For molecular biology applications, the extracted DNA needs to be of sufficient concentration and of high quality[7].

In the field, larvae of black flies can be easily collected from substrates and their rearing in laboratory is complex. For this reason, larvae were stored in ethanol. The extraction of genomic DNA from ethanol stored specimen is challenging while using both traditional and advanced methods. In this study, we have solved this problem by simple modification of the protocol for example ethanol preserved samples were incubated at 50 °C for 30 min to facilitate the evaporation of the ethanol from the sample. Further, an adequate amount of lysozyme was added to yield high amount of DNA. On the contrary, while using commercial kits or traditional method, the protocol used low concentration of lysozyme given for general isolation of genomic DNA from insects. In such cases, the generalized protocol for the isolation of genomic DNA sample did not yield good concentration of the DNA from the ethanol preserved

samples[14].

Furthermore, this study shows the method for isolates DNA of substantial quantity and high quality for use in PCR reactions[15]. The added advantage is that the procedure is suitable for a range of insect species in different life-stages and preservation methods. The most important feature of the proposed method lies in the fact that it does not require specialist equipment other than a centrifuge[6]. While many other high-throughput extraction methods published so far can offer similar or better efficiency, and they usually rely on some type of non-standard equipment, like sonicators, mills or robots[16-22]. In other cases, expensive deep-well plates are required. The cost of purchasing such non-standard items may be particularly prohibitive for researchers from developing countries, small laboratories and field stations[6]. The purpose of this method is to establish an economical, simple and rapid method for extracting genomic DNA from ethanol preserved insect samples. In future, the present methodology will be useful for students to learn basic and advanced molecular microbiological techniques.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This work was supported by Science and Engineering Research Board, Department of Science and Technology – Fast Track Young Scientists Project, Government of India (Ref. No. YSS/2014/000240/Dt. 06.11.2015).

References

- [1] Anbalagan S, Prasanna VA, Dinakaran S, Krishnan M. Two new species of *Simulium (Gomphostilbia)* (Diptera: Simuliidae) from Peninsular India with keys to Peninsular Indian members of the genus *Simulium*. *Zootaxa* 2014; **3861**(5): 451-65.
- [2] Anbalagan S, Prasanna VA, Ponraman G, Balachandran C, Dinakaran S, Krishnan M. Distributional pattern of aquatic insects in a hill resort region of South India with reference to tourism. *Int J Res Zool* 2014; **4**(2): 36-45.
- [3] Adler PH, Crosskey RW. World blackflies (Diptera: Simuliidae): a comprehensive revision of the taxonomic and geographical inventory; 2016. [Online] Available from: <http://www.clemson.edu/cafls/biomial/pdfs/blackflyinventory.pdf> [Accessed on 2nd May, 2016]
- [4] Figueiró R, Gil-Azevedo LH, Maia-Herzog M, Monteiro RF. Diversity and microdistribution of black fly (Diptera: Simuliidae) assemblages in the tropical savanna streams of the Brazilian cerrado. *Mem Inst Oswaldo Cruz* 2012; **107**(3): 362-9.
- [5] Anbalagan S, Bharathiraja C, Pandiarajan J, Dinakaran S, Krishnan M. Use of random amplified polymorphic DNA (RAPD) to study genetic diversity within a population of blackfly, *Simulium graveleyi* from Palni Hills, Peninsular India. *Biologia* 2012; **67**(6): 1195-203.
- [6] Milligan BG. Total DNA isolation. In: Hoelzel AR, editor. *Molecular genetic analysis of population: a practical approach (practical approach series)*. 2nd ed. Oxford: Oxford University Press; 1998, p. 29-64.
- [7] Kannan M, Suganya T, Arunprasanna V, Rameshkumar N, Krishnan M. An efficient method for extraction of genomic DNA from insect gut bacteria – culture dependent. *Curr Res Microbiol Biotechnol* 2015; **3**(1): 550-6.
- [8] Lagisz M, Port G, Wolff K. A cost-effective, simple and high-throughput method for DNA extraction from insects. *Insect Sci* 2010; **17**(5): 465-70.
- [9] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. *Current protocols in molecular biology*. New York: John Wiley and Sons; 1995.
- [10] Shokralla S, Singer GA, Hajibabaei M. Direct PCR amplification and sequencing of specimens' DNA from preservative ethanol. *Biotechniques* 2010; **48**(3): 233-4.
- [11] Asghar U, Malik MF, Anwar F, Javed A, Raza A. DNA extraction from insects by using different techniques: a review. *Adv Entomol* 2015; **3**: 132-8.
- [12] Reineke A, Karlovsky P, Zebitz CP. Preparation and purification of DNA from insects for AFLP analysis. *Insect Mol Biol* 1998; **7**(1): 95-9.
- [13] Hajibabaei M, deWaard JR, Ivanova NV, Ratnasingham S, Dooh RT, Kirk SL, et al. Critical factors for assembling a high volume of DNA barcodes. *Philos Trans R Soc Lond B Biol Sci* 2005; **360**(1462): 1959-67.
- [14] Chen M, Zhu YY, Tao J, Luo YQ. Methodological comparison of DNA extraction from *Holcocerus hippophaecolus* (Lepidoptera: Cossidae) for AFLP analysis. *For Stud China* 2008; **10**(3): 189-92.
- [15] Houck MA, Clark JB, Peterson KR, Kidwell MG. Possible horizontal transfer of *Drosophila* genes by the mite *Proctolaelaps regalis*. *Science* 1991; **253**(5024): 1125-8.
- [16] Anbalagan S, Arunprasanna V, Kannan M, Dinakaran S, Krishnan M. *Simulium (Gomphostilbia)* (Diptera: Simuliidae) from Southern Western Ghats, India: two new species and DNA barcoding. *Acta Trop* 2015; **149**: 94-105.
- [17] Ashburner M. *Drosophila: a laboratory handbook*. New York: Cold Spring Harbor Laboratory Press; 1989, p. 106-7.
- [18] Gustincich S, Manfioletti G, Del Sal G, Schneider C, Carninci P. A fast method for high-quality genomic DNA extraction from whole human blood. *Biotechniques* 1991; **11**(3): 298-300, 302.
- [19] Ivanova NV, Dewaard JR, Hebert PDN. An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Mol Ecol Resour* 2006; **6**(4): 998-1002.
- [20] Hunter SJ, Goodall TI, Walsh KA, Owen R, Day JC. Nondestructive DNA extraction from blackflies (Diptera: Simuliidae): retaining voucher specimens for DNA barcoding projects. *Mol Ecol Resour* 2008; **8**(1): 56-61.
- [21] Hoarau G, Coyer JA, Stam WT, Olsen JL. A fast and inexpensive DNA extraction/purification protocol for brown macroalgae. *Mol Ecol Resour* 2007; **7**(2): 191-3.
- [22] Whitlock R, Hipperson H, Mannarelli M, Burke T. A high-throughput protocol for extracting high-purity genomic DNA from plants and animals. *Mol Ecol Resour* 2008; **8**(4): 736-41.