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Suitability of the boiling method of DNA extraction in mosquitoes for routine molecular analyses

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ABSTRACT
This communication deals with the experience on suitability of the boiling method of DNA extraction from mosquito tissues. The DNA extracted by this method was found, by and large, stable after 30 months of storage. The method is useful for routine molecular entomological applications.

Keywords: DNA, Extraction technique, Mosquitoes, PCR.

1. Introduction
Molecular tools such as polymerase chain reaction (PCR) and DNA sequencing are being used commonly to study mosquito systematics, taxonomy and evolutionary genetics of disease vectors of public health importance. These investigations require collection of large numbers of target mosquitoes from the field and their DNA analyses in the laboratory. For downstream molecular analyses, *viz*., PCR, sequencing etc, isolation of good quality DNA in sufficient quantity is a fundamental requirement. To meet this requirement numerous DNA isolation protocols for microbes, invertebrates, vertebrates and plant tissues have been developed [1]. Most of the mosquito related DNA based studies rely on chelax based [2], phenol-chloroform based [3] and FTA card based [4] methods of DNA extraction. All these methods are good but require either specific reagents or equipments and also take a longer time posing operational challenge while dealing with a large number of samples. In certain research areas of molecular entomology like systematics, barcoding and taxonomy, it is highly desirable to identify the species without destroying the complete specimen so that the voucher specimen can be preserved [5]. Although some non-destructive DNA sampling methods are available for insects [6, 7], these techniques are sometimes found inefficient or require multiple steps of specimen preparation [6]. A simple, fast and inexpensive boiling method of DNA extraction from mosquitoes is also available [2]. Variants of this method though have been used in some mosquito studies [8, 9, 10], it is relatively a less preferred method due to the possibility of rapid degradation of DNA extracted by this method. Here we report our experience on the boiling method of DNA extraction from mosquitoes that we have found suitable for most of the routine molecular biology analyses, including sequencing, related to mosquitoes for immediate application as well as after its storage for nearly 3 years. We used fore legs of mosquito for DNA extraction as it contains fewer or no morphological information , very less pigments and PCR inhibitors [11].

2. Materials and Methods
A total of 115 *Anopheles dirus* complex adult mosquitoes were collected from various forested/forest fringed localities of the seven north-eastern states between 2007- 2010 using standard collection methods. Fore legs of the individual mosquito, stored singly in plastic beam capsule along with silica beads after collection, were excised aseptically, transferred to a sterile 0.6 ml micro-centrifuge tube and crushed carefully with the help of a sterile plastic pestle. It was homogenized thoroughly after adding 40 µl of 0.1 X Tris-EDTA buffer (1mM Tris-HCl, 0.1mM EDTA, pH 8.0). The homogenate was vortexed briefly followed by short spinning in a tabletop micro-centrifuge, incubated in a water bath maintained at 95 ºC for 8 minutes and finally centrifuged in a tabletop micro-centrifuge at 8000 rpm for 2 minutes.

The supernatant (~ 30 µl) was pipette out and stored in a fresh sterile 0.5 ml micro-centrifuge tube. Half of the supernatant was used for immediate application and the remaining was stored in a -20 ºC freezer. One µl of the supernatant, which contained DNA, was used for allele specific PCR (ASPCR) based species identification, amplification of nuclear loci such as r-DNA-ITS2, r-DNA-D3 as well as mitochondrial loci *viz*. COII and ND5 genes in a 25 µl reaction volume following PCR protocols mentioned elsewhere [12, 13, 14] and later used for sequencing of these loci. The primers and the cycling conditions are shown in Table 1.

Table 1: List of PCR Primers and thermal cycling conditions for various molecular analyses

Locus	Primers (5' - 3')	Cycling conditions	Reference
ASPCR	D-U: CGC CGG GGC CGA GGT GG	94^{O}C/5 min, (94 ^{O}C/1 min, 58 ^{O}C/1 min,72 ^{O}C/2 min)X35, 72 ^{O}C/7 min	12
	D-AC: CAC AGC GAC TCC ACA CG		
	D-B: CGG GAT ATG GGT CGG CC		
	D-D: GCG CGG GAC GTC CGT T		
	D-F: AAC GGC GGT CCC CTT TG		
ITS2	For: TGT GAA CTG CAG GAC ACA T	94^{O}C/5 min, (94 ^{O}C/1 min, 51 ^{O}C/1 min,72 ^{O}C/2 min)X40, 72 ^{O}C/7 min	12
	Rev: TAT GCT TAA ATT CAG GGG GT		
D3	For: CCTTTGTACACACGCCCCGT	94^{O}C/5 min, (94 ^{O}C/30 sec, 55 ^{O}C/30 sec,72 ^{O}C/1 min)X40, 72 ^{O}C/7 min	14
	Rev:GTTCATGTGTCCTGCAGTTCAC		
COII	For: ATG GCA GAT TAG TGC AAT GG	94^{O}C/5 min, (94 ^{O}C/1 min, 51 ^{O}C/1 min,72 ^{O}C/2 min)X40, 72 ^{O}C/7 min	13
	Rev: GTT TAA GAG ACC AGT ACT TG		
ND5	For: GGHYTAACTGTWWGTTATTCATTTC	94^{O}C/5 min, (94 ^{O}C/1 min, 51 ^{O}C/1 min,72 ^{O}C/2 min)X40, 72 ^{O}C/7 min	14
	Rev: CCMYAACATCTTCARTGTYAWRCTC		

3. Results and Discussion

The DNA extracted using the boiling method was used successfully for immediate application in ASPCR assays, for amplifying nuclear loci as well as mitochondrial loci, with the average yield obtained 15 ng/µl per amplicon, and also for sequencing. Fairly good amplifications of these loci were obtained with DNA stored for 30 months though weakening of the PCR product intensity was observed in about 15% samples amplified for nuclear loci, nearly in all samples amplified for mitochondrial loci (Fig. 1). Nearly 13% samples of the stored DNA failed to amplify in ASPCR assays.

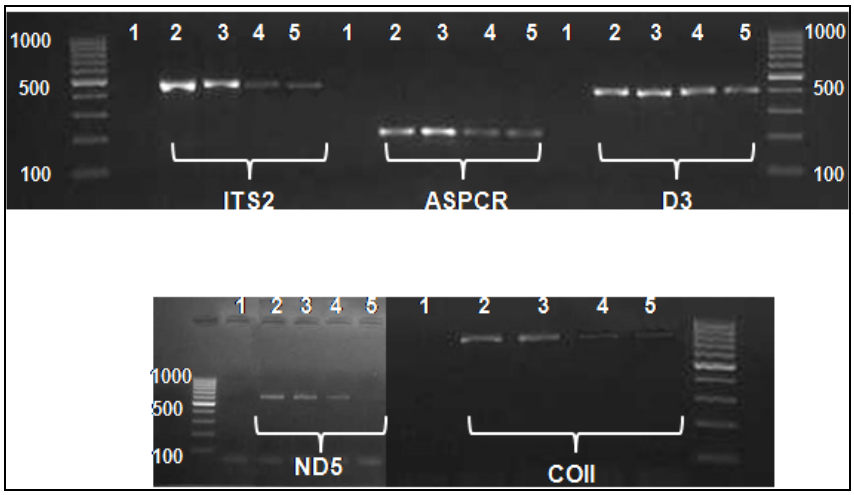

Fig 1: Gel images of the amplified PCR products for different nuclear and mitochondrial loci of *An. baimaii* DNA extracted using boiling method (lanes 2 & 3 fresh DNA, lanes 4 & 5 DNA stored for 30 months

The boiling method of DNA extraction yielded an average 7.42±2.28 SD ng/ µl of DNA (range 5.0-7.8) from the forelegs of *An. baimaii* mosquitoes (cf 4.8±1.19 SD ng/µl DNA yield by the standard Phenol-Chloroform method). The DNA quality, measured as A_{260}/A_{280} ratio in nanodrop 2000 spectrophotometer (Thermo Scientific, USA), was generally found good (mean 1.5±0.09 SD, range 1.43-1.64) but inferior to the Phenol-Chloroform extraction method (1.75±0.10 SD, range 1.65-1.89). Lower A_{260}/A_{280} ratio and higher yield of DNA, suggestive of a mixture of DNA and proteins in the product extracted using simple boiling method, was as per the expectation since no deproteination agent such as proteinase K was included in the extraction method.

DNA extracted by this method allowed amplification and sequencing of PCR products ranging ~ 200 bp (ASPCR) to ~ 850 bp (COII). The quality of DNA was reasonably good and DNA was found stable up to $2^{1/2}$ years on storage. The method is simple and rapid involving fewer steps, capable of handling large number of samples in a short span of time, does not need any special equipment and chemical (except a centrifuge and Tris-EDTA buffer) and does not contain any hazardous chemical in the extraction buffer. Importantly, this method is capable of extracting sufficient DNA even from one or two legs of a mosquito without destroying the complete specimen or its important morphological characters. These attributes make this method of DNA extraction suitable for laboratories handling large number of mosquitoes for routine molecular work such as systematics, DNA bar coding and disease transmission studies etc. especially where a very long term DNA storage is not required.

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