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PRESERVATION TECHNIQUES AFFECTING DNA EXTRACTION FROM *CIMEX LECTULARIUS* (HEMIPTERA: CIMICIDAE)

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Abstract The quality and quantity of deoxyribonucleic acid (DNA) extracted from insects are extremely important in genomic research. We tested the hypothesis that DNA can be extracted from bed bugs when stored in different preservatives at various temperatures. The bed bugs were stored in five preservatives including ethanol 90 and 70%, isopropanol 90 and 70%, and the no preservative. Bed bugs in all preservatives were stored at -80°C, -20°C and room temperature. DNA from bed bugs was extracted using Puregene Core Kit A. The DNA quantity was measured by Nanodrop-1000 and the DNA quality was determined by running 0.3 µg DNA in 1.7 agarose gel. We observed the significant reduction in the DNA yield over time. The DNA quantity ranged from 87.67 to 143.56 ng/µl, and DNA purity ranged from 1.82 to 2.1 (best purity = 1.80-2.00). Our data demonstrated that DNA can be extracted from bed bugs regardless of preservatives and temperatures. These data are exceedingly valuable to support genomic and forensic research.

Key words Bed bugs, genomics, molecular, isopropanol, ethanol.

INTRODUCTION

Insect genomic research on systematics, resistance management, forensic science, population biology and invasive pests has advanced in the recent years. Since the field collected insects are commonly used for the molecular study, appropriate handling and preservation of insect specimens are very important. Freshly killed insects yield the most DNA but it is impractical to maintain live insects in all situations. Insect DNA can remain intact when placed in appropriate preservatives and/or at certain temperatures. DNA of an organism stops replicating after death and degrades over time by factors such as light, oxygen and/or preservatives. In DNA studies, researchers have evaluated ethanol (Post et al., 1993; Dillion et. al., 1996; King and Porter, 2004; Frampton et al.; 2008, Shokralla et al., 2010; Moreau et al., 2013), DMSO solutions (Frampton et al., 2008), isopropanol (King and Porter, 2004), and -80°C (Dillion et. al., 1996; Frampton et al., 2008). Dillion et al. (1996) reported that two hymenopteran insects preserved in 100 % ethanol at -80°C yielded excellent DNA quantity and quality and can be amplified by Polymerase Chain Reaction (PCR) until 16 months. Freshly killed insects preserved in liquid nitrogen at -80°C can be very effective in maintaining DNA intact (Reiss et al., 1995) but this may not be always feasible. Post et al. (1993) got highest yield and less sheared DNA from blackflies when stored in liquid nitrogen or ethanol at room temperature. The best preservation media and the temperatures may vary from insect species to species. With recent resurgence of bed bug in the United States of America, few researchers are involved in genomic research to define population variations. Our research objectives were to determine if high quality of DNA from bed bugs can be extracted using different preservatives at different temperatures up to 24 months.

MATERIALS AND METHODS

The bed bug (*Cimex lectularius*) used in this experiment were reared in the laboratory, University of Nebraska Lincoln, NE, USA. The bed bugs were fed weekly on reconstituted human blood collected from the Nebraska blood bank (Lincoln, NE) and colonies were maintained in growth chambers at 23 ± 2°C, 55 ± 5% relative humidity, and a photoperiod of 14:10 (Light: Dark) (Montes et al., 2002).

We stored bed bugs using five preservative techniques at different temperatures (Table 1).

Table 1. Bed bugs specimens preserved in different preservatives and temperatures for DNA extraction.

	Preservatives				
	Ethanol		Isopropanol		No Preservative
Temperature	90%	70%	90%	70%	Not Applicable
-80°C	60*	60	60	60	60
-20°C	60	60	60	60	60
**RT(~24°C)	60	60	60	60	60

*Number of bed bugs preserved per treatment **RT = Room Temperature

Approximately 1,000 adult female bed bugs were placed in a glass container (6 cm x 15 cm) and set in a freezer at -20°C for 20 minutes for quick kill before assigning to each treatment. Sixty bed bugs were randomly transferred to individual glass vials (2 cm x 6 cm) containing respective preservatives thus totaling to 900 bed bugs. The experimental design was a complete randomized design.
Three bed bugs were withdrawn from the each glass vial representing the replicate for DNA extraction at intervals of week1, month(s) 1, 6, 12 and 24. For day 1, DNA was extracted from four bed bugs that were crushed immediately after killing.

DNA Extraction, Quantification, and Quality Check: The DNA was extracted from whole body of insects using Puregene Core Kit A (Qiagen, Valencia, CA, and Cat. No. 1042601). The DNA was eluted in 30 µl of the elution buffer provided with the kit. The quantity and purity of DNA was measured by NanoDrop-l,000 (Thermo Fisher Scientific, Pittsburgh, PA), and the quality was measured by running 0.3 µg of DNA per sample in 1.7 % agarose gel, stained with ethidium bromide, gel image visualized using UV irradiation and documented using Bio-Rad Gel Doc 2000 gel documentation system (Bio-Rad Laboratories, Hercules, CA). In NanoDrop-l,000, the purity of DNA was measured as the ratio of the absorbance at 260 nm by the absorbance at 280.

PCR Amplification: DNA from all 15 treatments were individually diluted to 15 µg/µl and 1 µl of DNA-dilution was used for PCR amplification using the *Taq* PCR Core Kit (Qiagen, Valencia, CA, and Cat. No. 201273) as per manufacture recommendations. Forward and reverse primers were designed using Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus) to amplify the gene *Actin* (Accession No.: LOC106674067), which is a highly expressed in the insects; its proteins are highly conserved proteins and are involved in cell motility, structure, and integrity. The primer used were Forward 5'CAGGGAAAAGATGACCCAG-3' and Reverse 5'TACCGATGGTGATGACCTGA-3'. The 7 µl of PCR product per sample were run in 1% agarose gel, stained with ethidium bromide, gel image visualized using UV irradiation and documented using Bio-Rad Gel Doc 2,000 gel documentation system (Bio-Rad Laboratories, Hercules, CA).

Data Analysis: We analyzed DNA data as repeated measures AVOVA ($P < 0.05$) using PROC GLIMMIX (SAS 9.4. SAS institute, NC, USA.). Means were separated by time for significant differences between extraction intervals and compared with Tukey Kramer's Method.

RESULTS

DNA Purity and Yield: Purity and the quantification of the DNA were done based on optical density measurement through spectrophotometer. The measurement of the optical density of 260/280 gives the purity; value ranging from 1.8-2.0 indicates the pure DNA. The value below 1.8 indicates tcontamination of DNA with phenol or other contaminants that absorb strongly at or near 280 nm. Purity of DNA from our extraction ranged from 1.82-2.10 indicating an excellent purity. The gel image of the DNA showed clear and distinct bands until 6 months, but we observed sheared DNA in latter intervals (Figure 1).

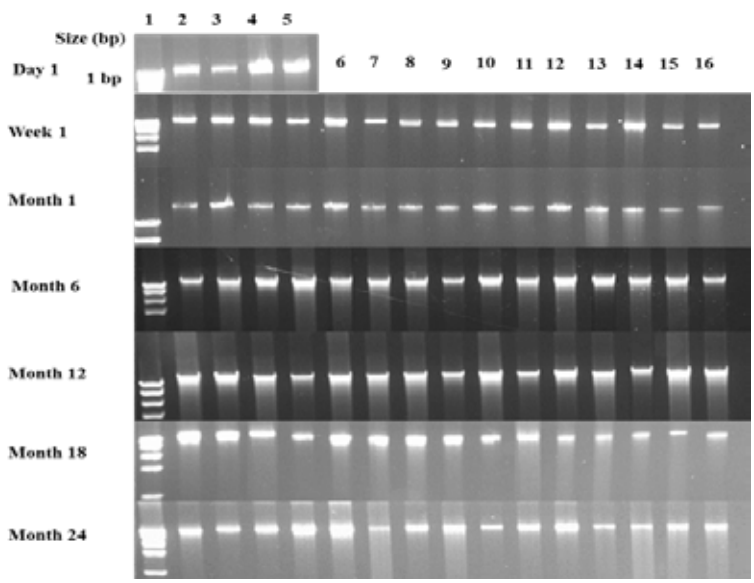

Figure 1. Genomic DNA of bed bug (0.3 µg/well) extracted from different treatments and ran on 1% agarose gel. Lane 1: DNA mass ladder, 2: Ethanol 90 (-80°C), Ln 3: Ethanol 70 (-80°C), 4: Ethanol 90 (-20°C), Ln 5: Ethanol 70 (-20°C), Ln 6: Ethanol 90 (room temp.), Ln 7: Ethanol 70 (room temp.), Ln 8: Isoproponal 90 (-80°C), Ln 9: Isoproponal 70 (-80°C), Ln 10: Isoproponal 90 (-20°C), Ln 11: 1sopropona1 70 (-20°C), Ln 12: Isoproponal 90 (Room Temp.), Ln 13: Isopropona 70 (room temp.), Ln 14 : No Preservative (-80°C), Ln 15: No Preservative (-20°C), Ln 16: No Preservative (room temperature).

The data analysis of DNA yield revealed significant effect of time (F= 23.08, df= 5, 140.9, P < 0.0001) (Figure 1). Average DNA yields at 18 and 24 month intervals were significantly lower when compared to DNA extracted at earlier intervals. Increase in the time lowered the DNA yield for all the preservatives and temperatures. In addition, we also observed the significant interaction between the treatments and time (F= 2.08, df =70, 130.6, P <0.0002). However, there was no significant effect of treatments (F=1.11, df= 14, 47.66, P > 0.05). The Average DNA yield for day 1 is 134.1µg/nl and the remaining DNA yields at different intervals are presented in Figure 2.

DISCUSSION

This is the first study to show the long term storage (up to 2 years) of bed bugs and successful isolation of DNA for PCR amplification. Ethanol and isopropanol are the most commonly used insect preservatives (Dillon et al., 1996; King and Porter, 2004). The alcohol concentrations of 95-100% are the most effective insect preservation for DNA isolation (Moreau et al., 2013) because ethanol enter the cell membrane barriers, deactivate the DNase activities, and also kills the microorganisms present (King and Porter, 2004). Ultra-cold storage temperatures are also commonly used for insect preservation (Frampton et al., 2008) because enzymes and antibodies lose much of their functional activity (Mandrioli et al., 2006). The factors critical to insect preservation are moisture, light and oxygen. High moisture content favors the microorganism growth and decay. Protein and nucleic acid degradation over time can be minimized by lowering temperature and avoiding light that triggers oxidation process (Prendini, et al., 2002). Bed bugs stored in glass vials at room temperature were not significantly different from those stored in ethanol or isopropanol and maintained at ultralow cold temperatures.

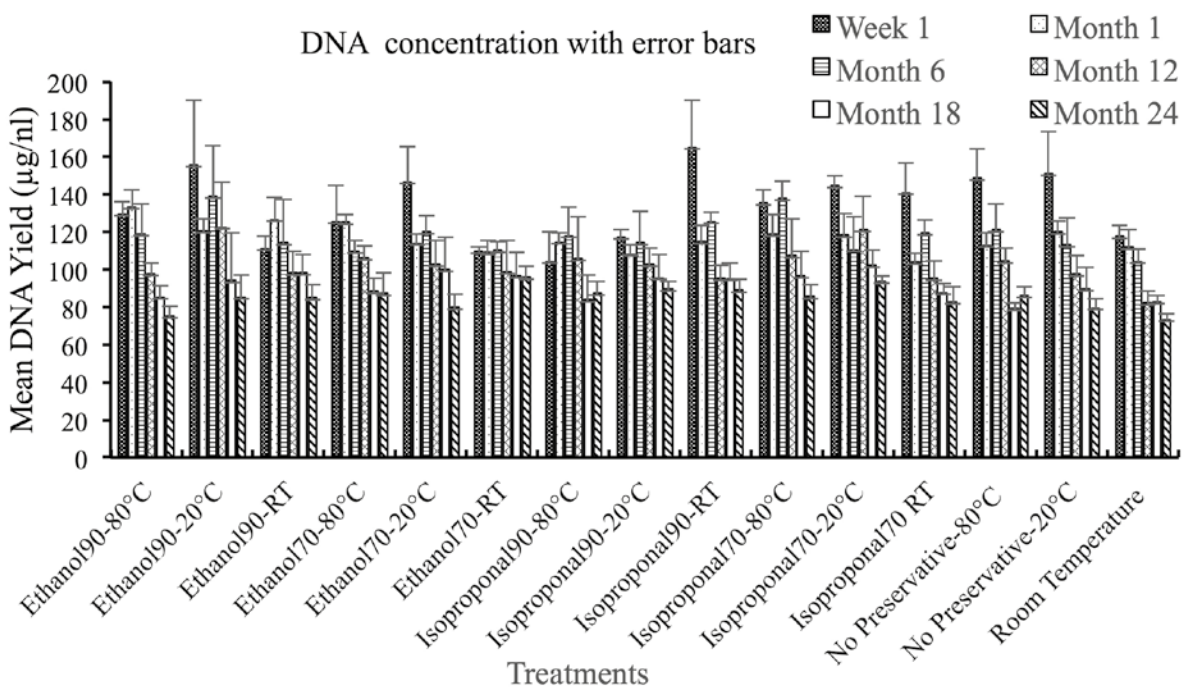

Figure. 2. Average DNA yield as measured by Nanodrop from different treatments. Each treatment (total samples =270) was replicated three times and average mean and the standard error were calculated.

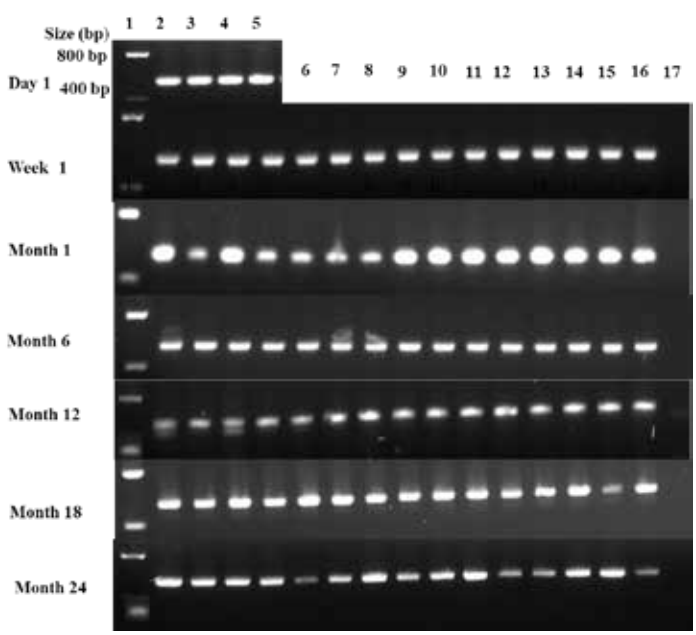

Figure 3. PCR product (487 bp) for *Actin* gene amplified from bed bug DNA Lane 1: DNA mass ladder, 2: Ethanol 90 (-80 °C), Ln 3: Ethanol 70 (-80°C), 4: Ethanol 90 (-20°C), Ln 5: Ethanol 70 (-20°C), Ln 6: Ethanol 90 room temp.), Ln 7: Ethanol 70 (room temp.), Ln 8: Isoproponal 90 (-80°C), Ln 9: Isoproponal 70 (-80°C), Ln 10: Isoproponal 90 (-20°C), Ln 11: 1sopropona1 70 (-20°C), Ln 12: 1sopropona1 90 (room temp.), Ln 13: Isopropona 70 (Room Temp.), Ln 14 : No Preservative (-80°C), Ln 15: No Preservative (-20°C), Ln 16: No Preservative (room temp.), Ln 17: Negative control.

The bed bugs have at room temperature (~24°C) in complete dark remains intact for up to 24 months. Lindahl (1993) reported that the degraded-DNA were extracted from the preserved termites under sterile laboratory conditions. Unlike these results, we found good quality DNA in bed bugs until 12 months. Our result showed DNA is well preserved until 6 months irrespective of preservation methods. However, we observed increase in smear due to the degradation of DNA in agarose gel after 6 months (Figure 1). In conclusion, our findings suggest that bed bugs can be stored at room temperature in glass vials until two years without any preservatives for DNA isolation and PCR amplification. These findings help to minimize the confusion of using preservatives and low temperatures for bed bug specimens intended for DNA based molecular research.

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