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Effect of different preservatives, temperatures, and storage durations on DNA extraction of Ladybird Beetles (Coleoptera: Coccinellidae

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Abstract *– High quality DNA for molecular studies can be easily extracted from fresh specimens. However, live samples are difficult to keep for long periods thus preservation of specimens is required maintaining the quality, specially when they are collected and transported from remote locations. In order to establish an effective method to preserve Ladybird beetles DNA for molecular studies, seven different preservation treatments with different concentrations of ethanol; 95%, 75% at controlled temperatures; – 80^{0}C (Ultra Cold freezing), 4^{0}C and room temperatures (RT) were compared. DNA was extracted from specimens kept in above treatments at three different time periods; 90, 180, 270 days, to analyze the effects of ethanol concentration, temperature and storage durations on quality and quantity of DNA. DNA extracted from fresh specimens was used as control. All treatments were efficient to preserve DNA except insects stored at 75 and 95% ethanol at room temperature, extracted at 270 days, which resulted in gel electrophoresis profile with missing bands. Ultra Cold temperature was the best temperature for the storage even in the presence or absence of ethanol. 95% and 75% ethanol at 4 ^{0}C and room temperature were the second best preservatives for lady bird beetles which had long period of storage up to 180 days with high quantity extracted DNA. In this paper it is proposed that 95% ethanol is the best concentration of ethanol to use as the preservative for maximizing the quantity and quality of DNA, as well as for maintaining morphological integrity when ultra-cold freezing is not immediately available.*

Keywords*: Insect, Ladybird beetle, DNA extraction, Preservatives, Storage period*

1 INTRODUCTION

Coccinellids (Coleoptera: Coccinellidae) is a highly diversified family with number families, tribes, and genera. Coccinellids are well recognized because of their use as biocontrol agents and this reason has given rise to the subject of many ecological studies throughout the world. As a tropical country Sri Lanka is rich in the species of Coccinellids specially in the areas where crop cultivation take place. Therefore further studies on their evolution might help on understanding the predatory pattern. However, little is known concerning phylogenetic relationships of the Coccinellidae, and a precise evolutionary framework is still required for the family. At present several researchers interest about studying molecular phylogeny leading to a new era of predatory world.

Molecular phylogeny which is popular among researchers, provide vast quantities of information for phylogenetic inference and taxonomic identification. It is obvious that

obtaining and collecting fresh material is time-consuming, expensive, and often fails to provide a wide coverage of the species (Houdt, et al, 2010). The development of new techniques in molecular genetics carried out by researchers assessing how field collection techniques and preservation practice effects on the condition of the specimens DNA (Post, et al. 1993, Reiss, et al. 1995, Austin and Dillon, 1997; Vogler and Pearson, 1996, Dean and Ballard, 2001, Cor, et al. 2005, Frampton,, et al. 2008). However, several evident limitations exist when using DNA from museum or old specimens; obtaining sufficient amounts of high-quality DNA is a major challenge. Hence, DNA quality use for amplification effected by age, method of storage as well as method of preservation (Van Houdt, et al, 2010). Results of earlier studies indicated that different pretreatment methods can significantly impact the purity and concentration of DNA extracts from dried or old insect specimens (Pu et al, 2002; Zang et al, 2004; An, et al, 2010; Li, et al, 2015).

In the present study it was focused on fresh specimens of Coccinellids stored in ethanol under different temperatures and range of durations for DNA extraction. The objectives of this study were to identify a best-practice approach for storage of high-quality DNA extracts from old specimens, and to test whether ethanol stored specimens can be used successfully for determining reproducible phylogenetic relationships.

3 METHODOLOGY

3.1 Treatments

Seven different preservation treatments, at controlled and room temperatures (RT), were compared: (1) -80 ^{o}C (Ultra cold temperature); (2) 95% ethanol at ultra-cold temperature; (3) 95% ethanol at 4 ^{o}C; (4) 95% ethanol at RT; (5) 75% ethanol at ultra-cold temperature; (6) 75% ethanol at 4 ^{o}C (7) 75% ethanol at RT. DNA extracted from fresh specimens was used as control. In all treatments, specimens were stored for 90, 180 and 270 days prior to DNA extraction. All specimens were kept in darkness for the duration of the experiment. Live *Monechilus sexmaculata* were collected from green houses in South China Agricultural University, Guangzhou and grew in chambers with controlled atmospheric conditions. For all treatments thirty individuals (ten per each storage time) were directly placed in 1.5 ml of preservative for each specimen. For all treatments the ratio of tissue volume to preservative volume was ~1:8.

3.2 DNA Extraction

Abdominal or thorax tissue from each single specimen was used for DNA extraction. An individual beetle was taken from the stock, soaked overnight in 1.5 ml TE buffer (pH 8.0) and dissected to separate the thorax or abdominal tissues. DNA was extracted according to the standard protocol of insect DNA extraction. The selected tissues were crushed in 10 µl STE buffer (10 mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0), and 0.15 mM Nacl) added with Protenase K (200 µg/ml), 1:100. The homogenate was digested with another 190 µl of STE and Protenase K buffer at 56 ^{0}C for 3 - 4 hours. In order to digest completely, the mixture was inverted several times during digestion. After digestion homogenate was added with equal volume of Phenol, mixed ten minutes, and centrifuged 12,000 rpm for 4 minutes. Supernatant was transferred to a new 1.5 ml tube and added with Phenol: Chloroform, 1:1, mixed and centrifuged 12,000 rpm, 4 minutes. Chloroform: Isoamyl alcohol, 24:1 added to the transferred supernatant to a new 1.5 ml tube and centrifuged at 12,000 rpm, for 4 minutes. Supernatant was precipitated overnight at -20 ^{0}C added with 1/10 of 3 M NaAc and double amount of total volume of absolute alcohol. Washed the

DNA pellet with 70% Ethanol two times, centrifuged at 12,000 rpm, 15 minutes at each time.

Properly dried DNA pellet was resuspended in 30 µl TE buffer and preserved at 4 ^{0}C. DNA was quantified at 2:100 dilutions with a Gene Quant II RNA/DNA calculator spectrophotometer (Pharmacia Biotech, Cambridge, UK) at 260 nm wavelength for determination of double-stranded DNA. In order to compare the results, crude DNA was gel electrophoresis and the bands observed were classified into three categories: no band, a very faint band and a very thick band (0, 1 and 2 respectively).

3.3 Experimental design

The statistical analysis of the data on the amount of DNA was made using a completely randomized split-plot design with 10 replications, where the preservation methods were the main plots and storage periods were the subplots. Significance of the electrophoresis results was evaluated using repeated measures within categorical modeling (CATMOD) in SAS (CATMOD; SAS Institute Inc. 1999) at α=0.05. CATMOD bases significance on a chi-squared statistic.

4 RESULTS

4.1 Amount of DNA extracted

The amount of DNA extracted from *Menochilus sexmaculata* by different preservation methods shown in Table 01. There was no significant effect of storage period on the amount of DNA extracted (F=0.14, df=2 and p=0869) but significant differences were observed for preservatives (F=6.28, df=7 and p<0.0001) as well as interaction between storage periods and storage methods. Specimens stored at 75% and 95% ethanol at room temperature consistently had lower yield of DNA in all storage periods, both of these treatments differed significantly from other preservation methods having a detrimental effect upon DNA yield, specially after 270 days (Table 01).

Table 01: DNA yield (ng)/insect extracted from *Menochilus sexmaculata* at 90, 180 and 270 days of storage under various combinations of temperature and ethanol concentrations

Treatments	Storage period (days)			Mean
	90	180	270	
-80 ^{0}C	116.91	126	107.2	**116.84 ab**
Ethanol 95% at -80 ^{0}C	116.55	151.76	107.14	**125.15 ab**
Ethanol 95% at 4 ^{0}C	99.92	102	98.78	**100.23 bc**
Ethanol 95% at RT †	43.91	44.92	33	**40.61 d**
Ethanol 75% at – 80 ^{0}C	108.75	116.25	26	**83.93 bc**
Ethanol 75% at 4 ^{0}C	61.37	61.02	50.2	**57.53 c**
Ethanol 75% at RT †	29.07	24.22	19.76	**24.35 d**
Fresh specimens	136.70	113.22	253.27	**167.73 a**

Mean	**89.14**	**92.42**	**86.91**

RT † - Room Temperature.

4.2 The effect of temperature on extracted DNA

The effect of temperature on the preservation can also be observed clearly by comparing all the preservation methods. DNA yield extracted under different preservative concentrations, temperature and three different durations are given in table 01. Although the DNA yield was statistically different for all the treatments, the amount of DNA obtained at ultra-cold temperature and 4 ^{0}C was higher than the DNA yield obtained at room temperature (Table 01). Ultra cold temperature was the best for the storage even in the presence or absence of ethanol. The amount of DNA extracted after storage at ultra-cold temperature, 95% ethanol at ultra-cold temperature and 75% ethanol at ultra-cold temperature were 116.84, 125.15 and 83.93 µl respectively.

4.3 The effect of storage period on extracted DNA

Considering the overall mean of three storage periods maximum DNA yield was observed for fresh specimens having a mean value of 167.73 µl of DNA. Apart from this the amount of DNA extracted from treatments, ultra cold temperature and 95% ethanol at ultra-cold temperature was statistically similar having mean values of 116.84 and 125.15 µl of DNA respectively. DNA yields obtained from 95% ethanol at room temperature and 75% ethanol at room temperature were also statistically similar giving 40.16 and 24.35 µl of DNA which was the lowest DNA yield form all treatments.

4.3 The effect of preservation method on extracted DNA

The influence of different preservation methods on DNA quality (considering the quality of bands in gel electrophoresis of crude DNA extracts) are shown in table 02. Highly significant effects of preservation methods ($\chi 2$= 56.5, $p<0.0001$) and interaction between storage period and preservation methods ($\chi 2$=43.97, $p<0.0001$) were observed on DNA quality. All the preservation methods of DNA were almost same quality after 270 days when compared to the control except in the treatments of 95% ethanol at room temperature and 75% ethanol at room temperature. The quality of DNA as the presence or absence of bands in gel electrophoresis as a percentage, isolated from *Menochilus sexmaculata* after storing under different conditions for several periods given in Table 02.

Table 02: Quality of DNA (presence or absence of bands in gel electrophoresis as a percentage) isolated from *Menochilus sexmaculata* after storage under different conditions for several periods.

Treatments	Storage period (days)			Mean***
	90	180	270	
-80 ^{0}C	100%(10/10)	100%(10/10)	100%(10/10)	**100%(30/30)**
Ethanol 95% at -80 ^{0}C	80%(8/10)	100%(10/10)	100%(10/10)	**93%(28/30)**
Ethanol 95% at 4 ^{0}C	80%(8/10)	100%(10/10)	100%(10/10)	**93%(28/30)**
Ethanol 95% at RT†	40%(4/10)	30%(3/10)	10%(1/10)	**26%(8/30)**

Ethanol 75% at – 80 ^{0}C	90%(9/10)	90%(9/10)	100%(10/10)	**93%(28/30)**
Ethanol 75% at 4 ^{0}C	60%(6/10)	40%(4/10)	100%(10/10)	**66%(20/30)**
Ethanol 75% at RT†	30%(3/10)	40%(4/10)	20%(2/10)	**30%(9/30)**
Fresh specimens	100%(10/10)	100%(10/10)	100%(10/10)	**100%(30/30)**
Mean	**72%(58/80)**	**75%(60/80)**	**78%(63/80)**	

RT† - Room Temperature

As per the table 02 quality of DNA produced was satisfactory, however; still there was an increase in DNA degradation in all preservation methods after 270 days of preservation. Samples preserved by using ultra cold temperature, 95% ethanol at ultra-cold temperature, 95% ethanol at 4 ^{0}C and 75% ethanol at ultra-cold temperature showed DNA (100, 93, 93 and 93% banding) quality almost similar to the control (100% presence of bands in PCR) whereas samples preserved by using 95% ethanol at room temperature, 75% ethanol at 4 ^{0}C and 75% at room temperature showed degradation in DNA quality when compared to fresh specimens. Specimens stored in 95% and 75% ethanol at room temperature showed unacceptable results for DNA quality producing 26% and 30% of banding at gel electrophoresis of crude DNA. Further, these results demonstrate that extracted DNA of specimens, preserved in 75% and 95% ethanol at room temperature is not suitable for further molecular studies. Table 03 explain the Quality of DNA (appearance of bands in gel electrophoresis as a percentage) isolated from *Menochilus sexmaculata* after storage under different conditions for several periods.

Table 03: Quality of DNA (appearance of bands in gel electrophoresis as a percentage) isolated from *Menochilus sexmaculata* after storage under different conditions for several periods

Treatments	Storage period (Days)	Appearance of bands %		
		0	**1**	**2**
-80 ^{0}C	90	0%3(0/10)	90%(9/10)	10%(1/10)
	180	0%(0/10)	50%(5/10)	50%(5/10)
	270	0%(0/10)	100%(10/10)	0%(0/10)
Ethanol 95% at -80 ^{0}C	90	0%(0/10)	50%(5/10)	50%(5/10)
	180	0%(0/10)	20%(2/10)	80%(8/10)
	270	0%(0/10)	50%(5/10)	50%(5/10)
Ethanol 95% at 4 ^{0}C	90	10%(1/10)	80%(8/10)	10%(1/10)
	180	0%(0/10)	100%(10/10)	0%(0/10)
	270	20%(2/10)	70%(7/10)	10%(1/10)
Ethanol 95% at RT†	90	60%(6/10)	40%(4/10)	0%(0/10)
	180	60%(6/10)	40%(4/10)	0%(0/10)
	270	100%(10/10)	0%(0/10)	0%(0/10)

Ethanol 75% at – 80 ^{0}C	90	10%(1/10)	60%(6/10)	30%(3/10)
	180	10%(1/10)	40%(4/10)	50%(5/10)
	270	0%(0/10)	90%(9/10)	10%(1/10)
Ethanol 75% at 4 ^{0}C	90	40%(4/10)	60%(6/10)	0%(0/10)
	180	40%(4/10)	60%(6/10)	0%(0/10)
	270	80%(8/10)	20%(2/10)	0%(0/10)
Ethanol 75% at RT†	90	70%(7/10)	30%(3/10)	0%(0/10)
	180	60%(5/10)	40%(5/10)	0%(0/10)
	270	80%(9/10)	20%(1/10)	0%(0/10)
Fresh specimens	90	0%(0/10)	20%(2/10)	80%(8/10)
	180	0%(0/10)	60%(6/10)	40%(4/10)
	270	0%(0/10)	80%(1/10)	20%(9/10)
Means		26.66% (64/240)	50.00% (120/240)	23.33% (56/240)

RT† - Room Temperature

5. DISCUSSION

There have been several studies on different insect species to try and determine the best taxon-specific preservative for preservation of DNA from specimens collected in the field, and although high-concentration ethanol has been shown to be a generally effective DNA preservation medium (Moreau et al., 2013 and Frampton et al., 2008), there are exceptions to that rule (Fukatsu, 1999 and Vink et al. 2005). Preservation of specimens in 75% and 95% ethanol at room temperature showed a detrimental effect on the amount of DNA extracted after 270 days of preservation. This decrease in the amount of DNA is similar to the findings of other researchers who experienced the same reduction in the quantity of DNA. Post et al. (1993) reported the reduction in DNA recovery with females of *Simulium damnosum* (Diptera: Simullidae) in 80% ethanol at room temperature when compared with 100% ethanol at 4 ^{0}C after 120 days of preservation. Similarly, Reiss et al. (1995) observed a significant decrease in the amount of DNA isolated from *Amara glacialis* (Mannerheim) (Coleoptera: Carabidae) stored in 95% ethanol at room temperature after only 73 days. Koch et al. (1998) also extracted low amounts of high molecular weight DNA, from the heads of *Simulium vittatum* Zetterstedt preserved in 80% ethanol at room temperature for approximately four years. Cor et al. (2005) found that DNA degradation occurs in tissue stored at room temperature over six weeks in 95% ethanol, and little degradation occurs after five days according to his personal observations.

According to the results of the present study, it is also preferable to store specimens at -80 ^{0}C and 4 ^{0}C. Preserving the quality of both genomic and mitochondrial DNA is of great importance for conducting molecular studies. It was found that freezing at –80 ^{0}C was the best method for killing and preserving specimens, but this is often impractical either because specimens cannot be captured alive or because –80 ^{0}C freezing facilities are not available. The use of ultra-cold freezing has been suggested by Post et al. (1993), Reiss et al. (1995), Dillon et al. (1996), Cor et al. (2005) and Frampton et al. (2008). It was also found that preservation in high percentage ethanol is the best if specimens are stored at -80 ^{0}C. Post et al. (1993) found that Diptera were best preserved at 4 ^{0}C in 100% ethanol, but they

did not tested specimen storage at -80 ^{0}C, Ultra cold freezing which may have produced better results. However, Moreau et al. 2013 found results incorugent to the present study that is 95% ethanol at room temperature as the best preservative for ant species collected in the field.

The results of our studies have shown that DNA extracted after preservation in 75 and 95% ethanol at room temperature may not be suitable for DNA analysis. These results are similar to the findings of Carvalho et al. (2000). They found that the reproducibility RAPD banding pattern from insect samples preserved in different preservatives at different periods of DNA isolation gave the same banding profile up to 210 days. However different banding pattern was observed with DNA extracted from specimens stored for 360 days in 95% ethanol at room temperature when compared to other preservation methods. The results also suggest that a possible DNA degradation of specimens preserved in alcohol which may be due to the continuing activity of nucleases, which could be inhibited with the addition of EDTA (Dessauer et al. 1990). According to the Frampton et al. (2008) preservative type and concentration affect the quality as well as the quantity of DNA that can be extracted from a given specimen. Additionally, some methods of preservation have adverse effects on morphological characters that need to be preserved for specimen identification.

6. CONCLUSION

According to the above findings, it can propose several storage methods that can be used for the storage of Ladybird beetle specimens for a longer period up to 9 months, aiming the isolation of high-quality DNA for further molecular analysis. Ladybird beetles can be well preserved in preservation methods preferentially in the order of: 95% ethanol at ultra-cold freezing at -80 ^{0}C, in ultra-cold freezing only at – 80 ^{o}C, ethanol at 4 ^{0}C, 75% ethanol at Ultra cold freezing at -80 ^{0}C and 75% ethanol at 4 ^{0}C. Preserving for a period of 9 months at 75 and 95% ethanol at room temperature cannot be accepted due to low DNA levels extracted. Although these results were based on *Menochilus sexmaculata*, it is entrusted that these results are potentially applicable to other arthropods and invertebrates. Further the authors believe that this will guide biologists in best choice of preservatives for preserving invertebrate tissues for current and future DNA based research.

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