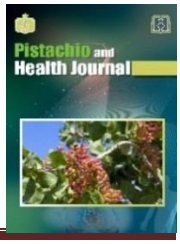

Assessment of Three Common Methods for DNA Extraction in *Trissolcus Sp*. Egg Parasitoid Wasp of Green Stink Bugs

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Abstract

Introduction: Extracting of genomic DNA with the proper quality and quantity is critical in the molecular identification of insects, whose morphological identification is problematic and affected by specific stage of life, size and sex. Also, storing insects under inappropriate conditions can have damaging effects on the quantity and quality of extracted DNA. Thus, choosing an appropriate protocol to provide pure DNA from these insects is essential.

Materials and Methods: In the present study to achieve an efficient method for DNA extraction from insect, we have applied and compared three common methods including (CTAB, Chelex and using commercial kit (animal tissue DNA isolation kit)). The quantity and quality of the extracted DNA were measured in two thermal conditions when DNA was stored at both -20 °C and -80 °C. The concentration of DNA extracted was measured on the Thermo Scientific NanoDrop 2000c.

Results: The greatest and lowest average nucleic acid concentration of parasitoids were recorded for CTAB and Chelex. The mean DNA yield of parasitoids (Scelionidae) from different collection preservatives indicated that the greatest average DNA yield was recorded for 95% and 99.8% ethanol respectively, and 75% ethanol had the lowest value of average DNA yield.

Conclusion: CTAB method as a suitable method. and storing insects in -80°C at 99.8% ethanol as suitable methods with high performance to DNA extraction of parasitoids was suggested.

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1. Introduction

Pistacia vera Linnaeus (Anacardiaceae) is one of the most important and valuable garden products in Iran. One of the important issues that today threatens the export of this product is the problem of pest existence. Green stink bugs (Pentatomidae) are very important due to quantitative and qualitative damage to pistachio crop. *Brachynema germari* Kolenati and *Acrosternum arabicum* Wagner are as two important species of green stink bugs [1-3]. Currently, the chemical control in pistachio area is the common method of controlling of theses bugs. The increasing use of pesticides and their irreparable losses has resulted in use of *biological pest control* [4]. Parasitoids are the most abundant and principal type of natural enemies due to the high diversity and parasitism in the host populations. Egg parasitoids, by reason of the attack on the first stage of pest life, has a high potential for biological struggle against pistachio bugs, so they are of particular importance in biological control programs [4-7]. Several individuals of parasitoids belong to the genera *Gryon*, *Psix*, *Telenomus* and *Trissolcus* of the family Scelionidae have played significant role in control of stink bug pests [4, 5, 8-10]. The accurate identification of natural enemies is the basis of biological control, the lack of suitable methods for identification can lead to failure of biological control programs. Most closely related parasitoids species have very small number of distinct morphological traits that used for accurate identification. Genetic and DNA-Based techniques have been used to identify species where morphological differentiation is problematic. The primary purpose to be considered is how to obtain high-quality DNA sample of insects. Sample DNA quality depends on the method of extracting, age and how to store [11]. The objective *of this research was to c*ompare common DNA extraction methods (CTAB, Chelex and DNA *extraction kit) and two thermal conditions* (-20°C and -80°C) for *preservation of wasps* in order to provide a simple, rapid and cost-effective method for DNA extraction of egg parasitoids (*Scelionidae).*

2. Materials and Methods

2.1. Stock Colonies of Green Stink Bugs

The adult insects of *Acrosternum arabicum* Wagner and *Brachynema germari* Kol were collected from the Rafsanjan pistachio orchards, the main pistachio area of Kerman province, Iran and they were held in plastic boxes (20×30×10cm) covered with mesh (0.5mm aperture) in growth chamber (27±1°C, 65±10% RH, and 16:8 [L:D] h photoperiod). They fed on diet of *Phaseolus vulgaris* L. (Fabales: Fabaceae) and *Salsola kali* L. (Caryophyllales: Chenopodiaceae) with water provided on a cotton wick. Eggs were collected daily from the boxes and one part of the fresh eggs was used for parasitism and the other part used for colony maintenance.

2.2. Stock Colonies of Parasitoids

The egg trapping technique was used to collect parasitoids. For this purpose, one-day-old eggs from laboratory stock colony stapled to yellow cards (7× 7 cm) and then attached to *S. kali* at a height of 50 cm in several different locations in pistachio orchards. The yellow

cards were collected after 48 hours and the parasitized eggs were placed in incubator with standard conditions (27± 1.0°C, 65± 10% RH, and 16:8 (L:D) h photoperiod) until adult wasps emerged. Parasitoids belong to *Scelionidae* family identified with identification key and reared on eggs of green stink bugs for experiments.

2.3. Storing Insects

The numerous fresh parasitoid wasps (*Scelionidae) and A. arabicum and* (Pentatomidae) were placed in 75%, 95% and 99.8% ethanol, and then stored at two thermal conditions (-20°C and -80°C) for six months.

2.4. DNA Extraction

Two common methods; Chelex and CTAB were adapted Walsh *et al*. [12] and Calderón-Cortés *et al*. [13] with brief modification and Kit method (animal tissue DNA isolation kit (S-1033-1), DNA Zit Asia company, Razavi Khorasan province, Iran) were used for DNA isolation of egg parasitoids and their hosts, green stink bugs. It should be noted, to obtain high-efficiency isolation and prevent cross-contamination TissueLyser (LT) should be employed, especially for egg parasitoids wasps.

2.4.1. Kit Method

DNA isolation were performed using animal tissue DNA isolation kit, according to the manufacturer instructions. Shortly, 180 µl preheated AT_1 buffer was added to the eppendorf tube containing of 10-15 mg tissue equal to 200-250 adults of wasps and nearly half of one bug. After vortexing, 200 µl AT_2 buffer was added and vortexed for 20 seconds. 20µl proteinase k was added and kept at 62 °C for 45 minutes. 0.5-1 µl of RNAase was added and incubated at 37°C for 15 minutes. 200 µl ethanol was added and the whole lysate was transferred to the spin column and tubes containing samples were centrifuged at 8000 rpm for 1 min. Then, 500 µl AT_3 and 700 AT_4 solutions was added separately to the columns and centrifuged at 10000 rpm for 1 min. Finally, the spin column was separated and transferred to the new microfuge tube and 50-100 AT_5 was added to the tubes and centrifuged at 10000 rpm for 1 min. The eluted solution at the bottom of microfuge tubes were contained genomic DNA. Samples were stored at −20°C for further processes.

2.4.2. CTAB Method

50 adults of egg parasitoid wasps were transferred to the 2ml eppendorf tube and pulverized with TissueLyser. 400µl Prewarmed (65°C) CTAB extraction buffer (0.02 M ethylene diamide tetraacetic acid (EDTA) pH 8.0, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 0.01% Sodium Metabisulfite ($Na_2S_2O_5$), 2% w/v CTAB) and 20µl proteinase k were added to the tubes. Tubes were kept at 62°C for 45 min with occasional shaking, and 1µl of RNAase was added to the solution and incubated at 37°C for 15 min. 400µl chloroform was added and centrifugation was done at 13,000 for 15 min. The supernatant (top aqueous) was transferred to the new microfuge tube and 400µl and 50µl ammonium acetate (C2H7NO2) were added to the tube. Tubes were centrifuged at 10000 rpm for 10 min. The top aqueous was discarded and 100µl of 70% chilled (-20°C) ethanol was added, tubes were centrifuged at 10000 for 10 min and the supernatant was discarded. The pellet was dried at room temperature for 1 hour. Finally, the DNA pellet re-suspended in

50 µl deionized water and was kept at room temperature for 30 min. Samples were stored at −20°C for further processes.

2.4.3. Chelex Method

50 adults of egg parasitoid wasps were transferred to the 2ml eppendorf tube and pulverized with TissueLyser. 100 µl Chelex-100 %5 and 5 µl Proteinase-K were added to the tubes. Then, 0.5 µl RNase was added to the solution and incubated at 37°C for 15 min. After that, tubes were kept at 100°C for 10 Minutes. Tubes were vortexed for 20 seconds and centrifuged at 10000 rpm for 10 minutes. The supernatural containing the DNA were transferred to the new tubes and stored at −20°C for further processes.

2.5. Data Analysis

Treatments were analysed using ANOVA and means were separated using the Tukey-Kramer honestly significant difference (HSD) test ($P<0.05$). Normality of data was done by the Kolmogorov–Smirnov test (K–S test). To compare means of concentration of DNA extracted when specimens stored at both -20 and -80 temperature, were accomplished with independent-samples t-test. Levene's test was also performed for checking equality of variances among treatments. Statistical analyses were conducted using (SPSS, v. 22).

3. Results

The results of the study found that DNA extraction methods significantly affected the DNA yield of parasitoids that the greatest and lowest average nucleic acid concentration were recorded for CTAB and Chelex ($P=0.000$, $F=41.730$, $df=14$). Similarly, CTAB had the highest value of average nucleic acid concentration for green stink bugs ($P=0.000$, $F=4.129$, $df=14$ and $P=0.043$, $F=41.002$, $df=14$) for B. germari and A. arabicum respectively (Table 1 and Fig. 1).

The results of purity ratios (260/280 and 260/230) of green stink bugs and parasitoid wasps presented in Table 1. The results of the mean DNA yield of parasitoides from different collection preservatives (75% ethanol, 95% ethanol and 99.8% ethanol) shown in Fig. 2.

The greatest average DNA yield was recorded for 95% ethanol and 99.8% ethanol, and 75% ethanol had the lowest value of average DNA yield ($P=0.000$, $F=36.719$, $df=14$). Two ultra low temperature as storage way had not significant effect on average nucleic acid concentration of egg parasitoid wasps (Table 2).

Table 1 The mean nucleic acid concentration and purity ratios measured with NanoDrop for Trissolcus sp., B. germari and A. arabicum.

Species	Methods of DNA extraction	DNA Concentraction (ng/µL)	260/280	260/230
Trissolcus sp	CTAB	213.86±19.95[a]	1.86	1.74
	Chelex	45.20±9.42[c]	1.27	0.62
	DNA Extraction Kit	124.46b±4.98[b]	1.84	1.80
B. germari	CTAB	229.64±57.54[a]	1.95	1.3
	Chelex	111.7±13.92[b]	1.18	0.67
	DNA Extraction Kit	100.74±14.37[b]	1.78	0.99
A. arabicum	CTAB	240.76±15.54[a]	1.97	1.51
	Chelex	90.24±13.13[b]	1.34	0.69
	DNA Extraction Kit	112.82±8.24[b]	1.81	1.12

Table 2- The mean nucleic acid *concentration for Trissolcus sp. measured with NanoDrop from two storage ways (Student's t-test, α=0.05)*

Temperature for DNA Storage	N	Mean	Std. Deviation	Std. Error Mean	T	df	Sig (2-tailed)
-20°C	5	113.54	16.99	7.59	-1.201	8	0.264
-80°C	5	124.46	11.15	4.98			

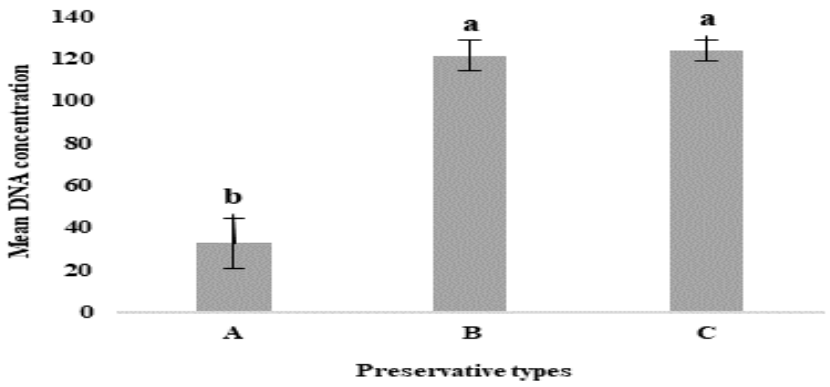

Fig. 2- The mean nucleic acid concentration for Trissolcus sp that measured with NanoDrop from different collection preservatives (A; 75% ethanol, B;95% ethanol and C;99.8% ethanol) (Tukey-Kramer HSD test, α=0.05).

4. Discussion

In the present study, Results revealed despite of that egg parasitoid wasp and pentatomid bugs (*A. arabicum* and *B. germari*) do not belong to the xylophagus insect but CTAB was appropriate method for DNA extraction of these genus. Asghar et al. [14] mentioned that xylophagus insects contain high concentration of phenolics, CTAB and modified CTAB-PVP method were used for DNA extraction. Also, Calderón-Cortés *et al.* [13] described CTAB-PVP method and introduced it a simple and rapid method for DNA isolation from xylophagous insects and concluded that DNA isolated by the CTAB-PVP method could be used in most molecular analyses. Other researcher used CTAB method in their work with some modified [15-18].

Currently, most commercial kits are available for DNA extraction. Some researcher used DNA extraction kits in their studies [19-24] due to obvious advantages such as 1- In addition to providing a rapid and efficient method for high-yield extraction of purified DNA from a variety of sample types, the reagents in DNA extraction kits are specially formulated to minimize the presence of contaminants and reduce nucleic acid degradation during preparation. 2- The easy-to-use, step-by-step nature of DNA extraction using a pre-formed kit also helps to minimize user-error thereby ensuring consistent isolation and minimal sample-to-sample variability.

Our finding indicates Chelex method was not appropriate for DNA extraction of egg parasitoids wasps and their hosts, pentatomid bugs. Asghar et al. [14] mentioned that DNA extraction of different insects, with various physical condition caused varying feasibility for Different techniques. They discussed and explained different methods with reasons of utilization: Chelex technique is more sufficient for DNA extraction of *insect vectors* by reason of short time requirement and *Good-quality DNA*. Charge Switch and prepGEM techniques are equally productive for DNA extraction of preserved insect but for dry specimen with more than 5 years shelf life have high quantity of DNA with Charge Switch technique and prepGEM technique is more *faster, cheaper* for freshly preserved specimen (dry or wet).

Storing insects under inappropriate conditions can have a detrimental effect on the quantity and quality of extracted DNA [25]. In our study, the average nucleic acid concentration recorded for 75% ethanol, as collection preservatives was in nadir. The explanation results of other researcher supported our findings that the use of ethanol at <95% is not desirable because the water in insects dilutes the ethanol, which can result in degradation of DNA. If the insects are large, it is best to kill them in 100% ethanol and then throw away ethanol and replace it with fresh ethanol to reduce its dilution with endogenous insect water. Storage in methanol, chlorophyll, or low concentrations of ethanol does not maintain DNA well (26, 27). Also, Hoy [27] discussed invaluable points about storage and ideal killing methods: storage and ideal killing methods include placing insects in a freezer at very low temperatures, or in liquid nitrogen or dry ice. Quick killing reduces DNA damage by *endogenous* DNases. Alternative methods for killing and store include the use of *Pure ethanol – 100% ethanol* or 95%. However, it is not always possible to kill and store insects under optimal conditions in remote field sites. Other methods of killing and storage may provide useful DNA, although its quality and quantity may

be reduced. Totally, determination of DNA extraction technique is relying upon species under study, DNA extraction time, economical stander of technique due to reagents and equipment used for extraction and above all extracted DNA quality. Overall, researcher try to detect and develop novel cost affective techniques, with more rapid and accurate result.

5. Conclusions

The results of this study demonstrates that for storage and molecular identifying of parasitoid wasps in the short term, the CTAB technique and storage of wasps in 99.8% ethanol at -80 °C would be appropriate, but more research was should done with regard to other DNA extraction techniques and storage preservatives of egg parasitoids (scelionidae).

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Conflict of Interest

The authors declare no conflict of interest.

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