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Comparison of different DNA extraction methods for the molecular study of bark beetles (Coleoptera: Curculionidae, Scolytinae)

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ABSTRACT. Bark beetles are one of the most important pests in forests. Because of their small size and highly similar morphological characters, molecular approaches may be quite useful for a valid species determination. In this context molecular identification represents an accurate and modern method for species identification. The purity and high quantity of extracted DNA have important role in successful amplification of the target fragment of the genome. The aim of this study was comparing different DNA extraction methods in order to choose the highest quality and quantity of DNA extract for the identification of bark beetles. During the study bark beetles were collected from different parts of the North forests of Iran. Five different DNA extraction methods were performed and evaluated on individual specimen including Chelex, Phenol chloroform, CTAB, salting out and Lysis buffer in the laboratory. The quantity and quality of extracted DNA were measured by spectrophotometer and gel electrophoresis. The result of DNA quantity mean ranged between (23.6-579.7 ng/μl) and the mean quality which was measured by 260/280 ratio (0.9-1.8). The statistical analysis was done by SPSS software, revealing significant differences between extraction methods. The results suggested that Chelex and salting out showed the highest quantity of all used methods.

Key words: Bark beetle, DNA extraction, Forest, Spectrophotometer

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Introduction

Since many years, molecular studies attract high attention among scientists in worldwide. This is also true for entomological questions, e.g. a correct species identification of pest species (Chen et al., 2010). In this context, DNA barcoding represents the most popular approaches for the determination of species of insect, which based on a short part of the mitochondrial genome (Hebert et al., 2003). As consequence, DNA extraction plays an important role in DNA barcoding, and a variety of methods have been established to isolate DNA from the tissue of insect (Milligan, 1998). Two factors represent important parameters

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for the extraction of DNA extraction, namely the quantification and qualification of the DNA content. These factors are mainly influenced by the freshness of samples, the used part of the tissue and materials, which are used in DNA extraction method. As a result, a proper DNA extraction method should improve DNA yield, decrease inhibitors effects and contamination, and should be cost-effective but not time-consuming (Chen et al., 2010). Different DNA extraction methods usually use a variety of different buffer including a salt to separate the DNA from proteins, and detergent such as sodium dodecyl sulfate (SDS) to inactivate enzymes (Sambrook et al., 1989; Cheung et al., 1993; Black & Duteau, 1997; Palma et al., 2016), and ethanol or isopropanol to precipitate DNA from its solution (Waldschmidt et al., 1997; Chen et al., 2010). The most crucial aspect among different DNA extraction methods for insect identification that is based on polymerase chain reaction (PCR), however, is the purity of extracted DNA (Ball & Armstrong, 2008) which is mostly affected by the presence of proteins, polyphenol or other putative inhibitors of PCR (Demeke & Jenkins, 2010). Other limiting factors are the high costs of some commercial kits and the necessity of specialized laboratory equipment (Rohland et al., 2010; Petrigh & Fugassa, 2013). In the past, various studies compared different DNA extraction methods in order to evaluate the most effective one for a variety of different insect taxa (Rampelotti et al., 2008; Chen et al., 2010; Rahimi et al., 2013).

Bark beetles of the subfamily Scolytinae (Col: Curculionidae) are among the most important pests of forest in the worldwide (Wood, 1982; Lawrence & Newton, 1995; Hucler et al., 2015). This group of beetles comprises more than 6,000 described species in the world which attack weakened trees, feed on the phloem and cause high damage in the forest and orchards (Furniss & Carolin, 1977; Knizek, 2011). Furthermore, ambrosia beetles as a distinct group of these beetles play a relevant role as vectors of pathogens that feed on fungi and dispersed them through different trees (Wood, 1982; Pfeffer & Knizek, 1995; Carillo et al., 2012; Hucler et al., 2015). Based on these aspects, an efficient management and useful control method is crucial in early-stage species determination (Santini & Faccoli, 2015). On the other hand, bark beetle identification using morphological characters is highly difficult and time consuming due to their small body size and subtle difference between species (Chang et al., 2013; Amini & Hosseini, 2016). Molecular methods as fast and accurate method need high quality and quantity of DNA. There are many studies that investigated on molecular identification of bark beetles which used commercial kits to extract DNA. (Jordal & Kmabastad, 2014; Chang et al., 2013; Victor & Zunica, 2016). Although commercial column-based kits allow an extraction of high quality DNA in terms of quality and quantity, the price of such kits can be too high for numerous labs.

Nevertheless, molecular techniques and the evaluation of extracted DNA are essential in molecular studies to confirm the morphological identification of bark beetles. The main purpose of this study was to evaluate DNA yielded and the quality of five different DNA extraction methods and compare them to determine the most efficient protocol for DNA extraction in bark and ambrosia beetle identification.

Material and methods

Sampling beetles

Beetles of different *Scolytus* species such as *Scolytus rugulosus* (Müller, 1818) and *Scolytus pygmaeus* (Fabricius 1787) were collected from different parts of the North Forest of Iran. Samples were collected by direct observation with a knife and brush under the bark of

trees. Samples were moved to 1.5 ml tubes contained ethanol 96% and transported to the laboratory. Morphological identification was confirmed by the first author and then samples frozen in separate 1.5 ml tubes at -20 °C for further molecular analysis.

DNA extraction

After morphological identification, each identified bark beetle species was washed and cleaned by elution buffer (Hosseini, 2010). Total genomic DNA was extracted from the whole body of bark beetles. Each species individually was prepared for different methods. For each method, 10 specimens were used as replicates.

Method 1: The method was based on Chelex 5%. Each species individually was homogenized using a sterile plastic pestle in 1.5 ml tubes with 50 µl phosphate buffer saline pH 7.4. Samples were incubated at 56 °C for 4-5 h followed by adding 500 µl Chelex 5% (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and placed in the water bath at 94 °C for 15 min. After centrifugation at 13,000 g for 5 min, DNA was transferred to a new tube and stored at -20 °C (Hosseini, 2010).

Method 2: The method was based on CTAB. The tissue was homogenized in 50 µl phosphate buffer, mixed with 10 µl Proteinase K and 440 µl extraction buffer (0.1M Tris-HCl pH 8, 10Mm EDTA pH 8, 2% SDS). Then incubated at 58 °C for 1 h, mixed by 150 µl NaCl 5M and 65 µl CTAB solution, again incubated at 65 °C for 10 minutes. Next, a mixture of 700 µl chloroform: isoamyl alcohol (24:1) was added and centrifuged at 13,000 rpm for 20 min. The aqueous phase was transferred to a new microfuge tube and the chloroform: isoamyl alcohol step repeated. The DNA precipitated with 400 µl 100% isopropanol and incubated at 4 °C for 8 h. The precipitate centrifuged at 13,000 rpm for 30 min. The resulting DNA pellet was washed twice with 500 µl 100% ethanol. Finally, the pellet was air-dried and mixed by 200-500 µl TE buffer (Hosseini, 2010).

Method 3: This method was based on cell lysis by phenol-chloroform. Each species individually was homogenized in 200 µl extraction buffer (Tris-CL 1M, EDTA 0.5 M, 10% SDS, H₂O), Then 3 µl Proteinase K was added and incubated at 56 °C for 1 hour. After adding 200 µl phenol, the samples were centrifuged at 1,000 rpm for 5 min. The aqueous phase was transferred to a new microfuge tube, a volume of 200 µl chloroform was added to exit the residue and this step was repeated. 400 µl ethanol and 10 µl NaCl was added and centrifuged at 13,000 rpm for 20 min. Ethanol was removed by incubating at 65 °C for 2-5 min. The pellet was mixed by 200-500 µl TE buffer (Hosseini, 2010).

Method 4: This method was based on Salting out. Each species was homogenized by 140 µl of the extraction buffer (0.1 M EDTA, 0.05 M Tris), 17.5 µl of SDS 10%. and 2 µl of proteinase K which incubated at 55 °C for 6 hr. 2 µl of RNase was added after 2-3 minutes, 40 µl of NaCl (>6M) was added too and centrifuged at 14000 rpm for 30 min .The upper phase was moved to new tube, 200 µl of chilled isopropanol was added to precipitate DNA and centrifuged at 14000 rpm for 20 min .The obtained pellet washed with 500 µl of 70% EtOH, centrifuged at 14000 rpm for 10 min .Finally, pellet was dried and dissolved the DNA in 20 µl of sterile H₂O (Patwary et al., 1994).

Method 5: DNA was extracted based on Lysis buffer method. The sample was pinned and then put in the tube 1.5 contained 400 µl Lysis buffer and heated on 56 °C for 12 hours. Then added 60 µl proteinase K centrifuged. DNA precipitate by 500 µl isopropanol and centrifuged. The upper phase was removed. Next, 400 µl ethanol and 100 µl Ammonium acetate were added, followed by the addition of 50 µl TE (Longmire et al., 1997).

All experiments were done through handing set up. The estimated time and cost of each protocol is calculated separately and listed in Table 3.

Table 1. Summary of DNA extraction methods were used.

<i>DNA extraction method</i>	<i>DNA purity A260/A280</i>	<i>DNA yield (ng DNA/mg sample)</i>
<i>Salting out</i>	1.30-2.10	428.6-711.8
<i>Chelex 5%</i>	1.20-2.20	380-680.2
<i>CTAB</i>	1.01-1.80	110-256
<i>Phenol-chloroform</i>	0.60-1.50	112-368
<i>Lysis buffer</i>	0.20-0.70	16.2-20.4

Spectrophotometric analyses of DNA

The quality and quantity of DNA extracted for each method were evaluated using a spectrophotometer (NanoDrop, Technologies Inc.). The ratio of absorbance at 260 nm and 280 nm was used to evaluate protein contamination. The concentration and absorbance ratios at 260-280 nm were measured by using 1 μ l of each sample in the Spectrophotometer (ND-1000, USA) (Table 1)

PCR amplification, gel electrophoresis and sequencing

The obtained extracted DNA in different method was diluted to 20 ng/ μ l and amplified by polymerase chain reaction using the universal primers sequencing including C1-J-1718 (5'-GGAGGATTGGAAATTGATTAGT TCC-3') as forward primer and C1-J-2411 (5'-GCTAATCATCTAAAACTTTAATTCCWGT WG-3') as reverse primer (Simon et al., 1994). PCR thermal program started by denaturation at 94 °C for 2 min, followed by 35 cycles denaturing 94 °C for 1 min, annealing 56 °C for 1 min, extension at 72 °C for 1 min with the final extension at 72 °C for 5 min. PCR product was analyzed by agarose gel electrophoresis using 1.5% agarose gel. The DNA bands were visualized and the image was capture using UVITEC France device. PCR products (salting out and Chelex) were selected, purified by and sequenced at Bioneer (Korea) and the BMR Genomics service (Padua, Italy). The sequences registered under individual accession number in National Center for Biotechnology information. (JX416903, JX089345, JX089344, JX089347, JX089346)

Statistical analysis

All experiments carried out in 10 replicates, but not all of them extracted successfully. The obtained data of quantity and quality of five methods were analyzed by Tukey's Studentized Range Tests. Data were analyzed completely randomized design at 5% probability level by using SPSS16.0 Software (Chicago, IL, USA). Differences between means were considered statistically significant at the 95% confidence level ($P < 0.05$).

Results

In this study five different extraction methods (Chelex, CTAB, Phenol chloroform, Lysis and Salting out) were done successfully and the light pellet was observed except in Chelex one. Although the replication for all methods was the same, two method in some replicates no DNA was found. In all five methods important factors such as quantity, quality, time consuming and the cost were evaluated. According to the spectrophotometric results the highest and the lowest quantity belongs to Salting out (579.2 ng/ μ l) and Lysis (24.6 ng/ μ l) methods respectively (Fig. 1). The mean quantity of five different methods ranged between 24.6 and 579.7 ng/ μ l (Table 3) which show the highest in Salting out. Results showed the

highest and lowest quality belongs to Chelex (1.88) and Lysis (0.87) nm ratio respectively (Fig. 2). The Highest quantity belongs to Salting out which also showed high quality. The Salting out and Chelex methods showed high purity near 2.00, otherwise, the Lysis buffer method showed the lowest level of absorbance equal 0.8 (Fig. 2). Phenol chloroform and CTAB methods results were nearly the same and were not considerable. The results of statistical analysis showed that there is significant difference in quality and quantity between DNA extraction methods (p value <0.0001) (Table 2). As the time and cost consider as important factors in extraction method, comparison of time consuming among five methods indicate that the longest term method is Lysis method which takes 12 hours and the shortest time method belong to Phenol chloroform takes below 5 hours (Table 3). According to the results all methods are in low cost (Table 3). Although the most expensive methods were Phenol-Chloroform and CTAB, these methods cheaper than commercial Kit extraction. Due to the gel electrophoresis results extracted DNA were amplified only in Chelex and Salting out methods because no band observed in other methods (Fig. 3). Pure PCR Products of samples which were extracted by Salting out and Chelex methods were sequenced successfully, and the obtained chromatographs were clean and without considerable noise (Fig. 4). Comparison of important factors such as DNA quantity, quality, time consuming and cost effective proved that the best DNA extraction method for bark beetles are Salting out and Chelex ones for bark beetles extraction which showed high level in this evaluation such as cost effectiveness and yielding high quantity and purity of extracted DNA than other methods.

Table 2. Analysis of variance quantity and quality of different DNA extraction methods.

Source	DF	Quantity		Quality	
		F	Pr > F	F	Pr > F
DNA extraction methods	4	57.08	<.0001	22.98	<.0001
Error	41	-	-	-	-
C V		25.67860		19.05881	

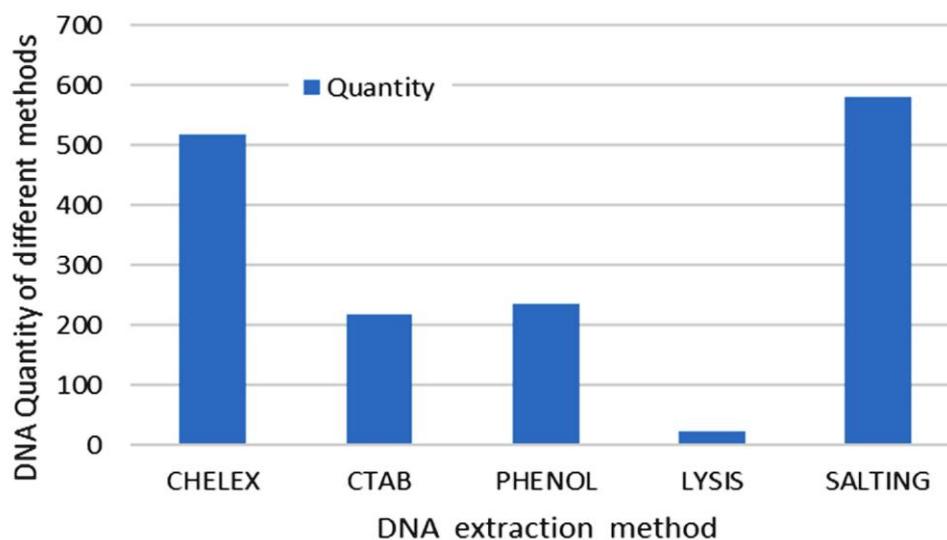


Figure 1. Mean level of bark beetle DNA extraction quantity (ng/ μl) of different methods.

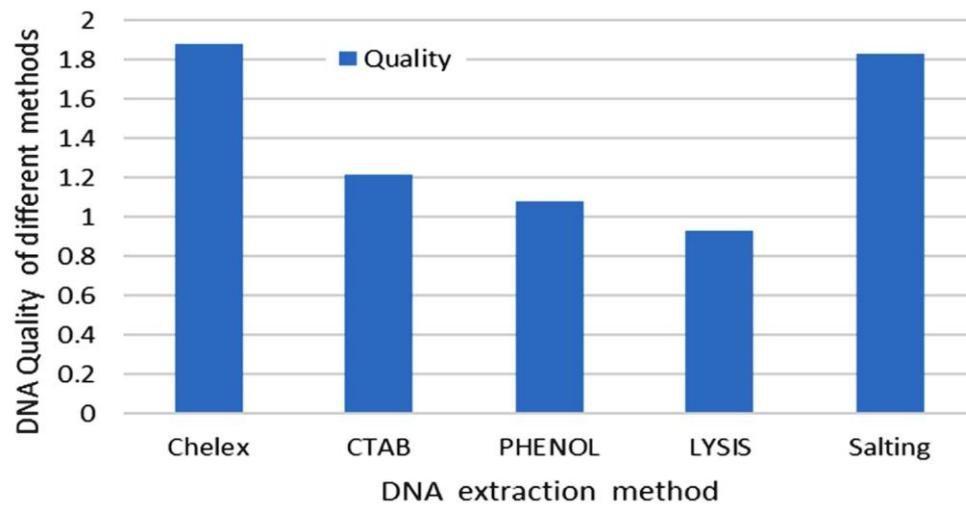


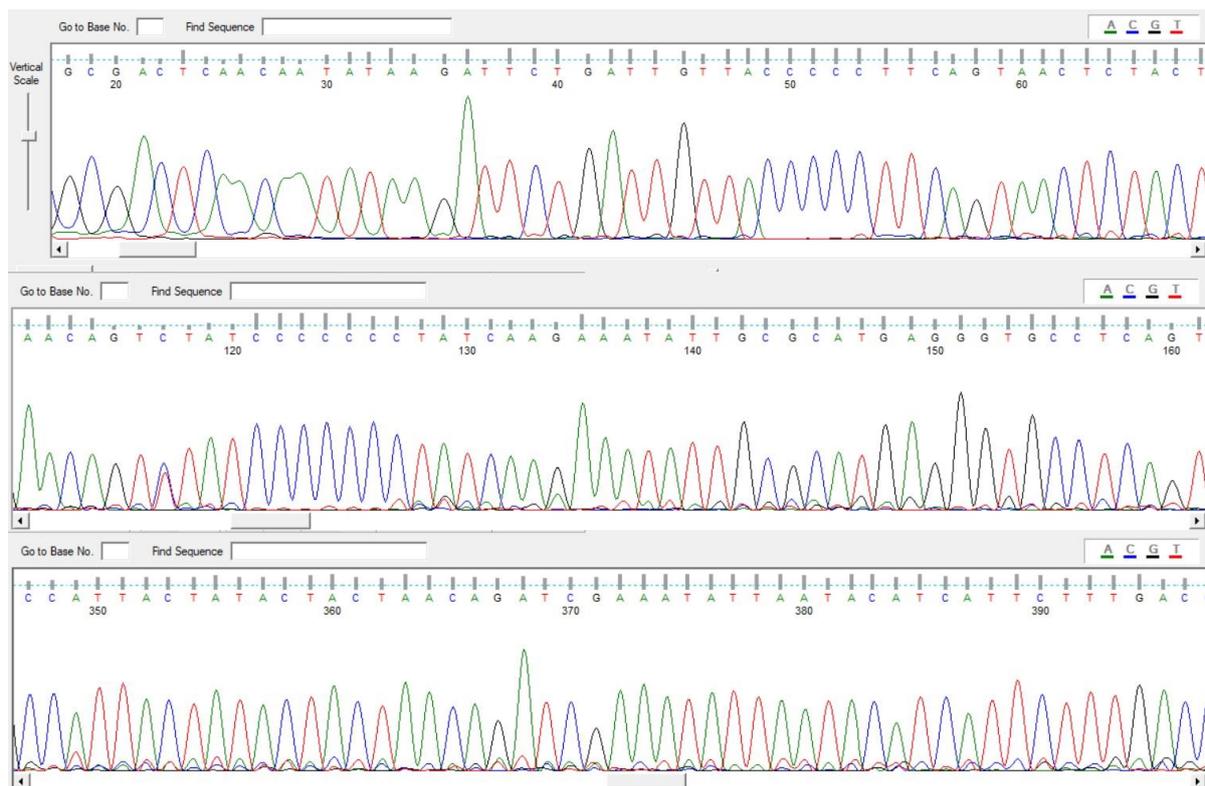
Figure 2. Mean level of Bark beetle DNA extraction quality (DNA/ Protein 260/280) of different methods.



Figure 3. Gel electrophoresis analysis of PCR product from five different DNA extraction methods.

Table 3. Comparison of important factors in five different DNA extraction methods.

<i>DNA extraction method</i>	Quantity (Mean)	Quality (Mean)	Time consuming (Hours)	Cost level per 100 samples ≈ \$
<i>Chelex</i>	518.7	1.88	5.2	17
<i>Salting out</i>	579.8	1.83	8	98
<i>CTAB</i>	219	1.21	10	100
<i>Phenol-chloroform</i>	234	1.07	2	130
<i>Lysis buffer</i>	24.6	0.87	12	80

**Figure 4.** Chromatograph of obtained sequences in Chelex and Salting out methods.

Discussion

Bark beetles are one of the most important pest groups in forest which cause high economic damage. Although accurate identification is necessary and is a crucial step in their management, bark beetle morphological characters is difficult to verify. Thus, DNA extraction would undoubtedly be applicable for a variety of molecular studies such as molecular identification, genetic diversity and phylogenetic. In the current study, five different DNA extraction methods were evaluated in quantity, quality, time-consuming and cost aspects. Although methods show a light color DNA pellet, there are some contaminations in Chelex ones, which leads to the dark color of DNA pellet. However this dark color pellet did not indicate the protein contamination because of high quality result. When the A260/A280 ratio which indicate the presence of protein is less than 1.6-1.8, means contamination with protein and/or polysaccharide of the insect tissue (Hosseini, 2010).

According to the results of the quantity and quality, the lowest quantity is belonging to the Lysis method which had been done without any detergent like SDS. Although Lysis method show low quantity and quality, it is a best choice for extraction samples which should not crushed and need vouchers to identify. However, the highest quantity was observed in Salting out which detergents such as SDS and chloroform were used. Although Chloroform was used to remove proteins and lipids substance (Hoy, 2003), methods such as CTAB, Lysis and Phenol chloroform extraction methods absorbance was below 1.8; therefore, the contamination of protein, salts, and polysaccharides which interfere in DNA amplification is nearly at high level in these methods. The findings of this research are consistent with previous studies (Pandey et al., 1996; Demeke & Jenkins, 2010). However, Salting out and Chelex methods show purity near 2.00 which is desirable for extracted DNA as was shown in other studies (Psifidi et al., 2010; Arif et al., 2010). Comparison to the previous studies, the lower contamination range observed in this study probably is due to the method of storing samples prior to the extraction. Although in most studies, the samples were maintained in liquid nitrogen and absolute alcohol, in this study samples were kept dry in freezer (-20 °C) with no preservative. In contrast to the most studies which use only a part of body such as leg or head for extraction, in this study the whole body of the beetle was extracted, although there are many phenolic and inhibitors substance in the stomach of beetles (Nancy et al., 2010).

Results revealed the highest quantity and quality of extracted DNA observed in salting out and Chelex methods. In these two methods detergents such as SDS and Chelex play an important role in obtaining high DNA concentration. According to the quality result, the Chelex and Salting out methods manifested the same quality level, but the quantity of the Salting out method was higher than Chelex. These two protocols showed a 260/280 nm ratio above 1.8, which is considered pure DNA (Dauphin et al., 2011; Psifidi et al., 2010). In terms of comparing time consumed in all methods, Phenol chloroform showed the low purity of extracted DNA, although it was the most rapid method. Whereas, the most time consuming method is the Lysis method which shows low quantity and quality. However, Salting out and Chelex methods which show high quantity and quality takes less than 10 hours which is considerable (Table 2). PCR reaction proved that only extracted DNA in Salting out and Chelex methods amplified successfully. Due to the quality of extracted DNA in Phenol chloroform method which was reported previously, the band was not observed as sharp as others to be considered. The clean and free of noises chromatograph proved the qualification of extracted DNA in salting out and chelex ones.

In spite of the fact that many DNA extraction commercial kits are available and commonly used in most molecular investigations to yield high DNA purity, but they are expensive and need professional equipment. The handy DNA extraction methods are cost-effective rather than using Kit, especially in developing countries. Based on the results of statistical analysis, different methods have different effect on DNA and all methods significantly yield DNA in different amounts, it is important to choose the most effective method. The Salting out and Chelex method found to be the most effective to obtain high concentration and purity of extracted DNA. Although these methods are more time-consuming than other evaluated methods, the differences time is not significant. Salting out and Chelex methods are cheaper, easier to use, safe and do not need any professional equipment for extraction than the commercial kits. This evaluation guided scientist choosing DNA extraction method for bark beetles molecular studies. Due to the importance of DNA qualification, the high concentration and purity of extracted DNA are essential in further molecular identification of bark beetles.

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Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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مقایسه روش‌های مختلف استخراج DNA در مطالعات مولکولی سوسک‌های پوست‌خوار (Coleoptera: Curculionidae, Scolytinae)

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چکیده: سوسک‌های پوست‌خوار یکی از مهم‌ترین آفات درختان جنگلی به شمار می‌روند. شناسایی سوسک‌های پوست‌خوار به دلیل اندازه کوچک و شباهت زیاد صفات ریخت‌شناسی نیازمند روش‌های مولکولی به عنوان یکی از کاربردی‌ترین روش‌ها است. مطالعات مولکولی به عنوان روشی دقیق و جدید برای شناسایی گونه‌های حشرات به کار می‌رود. استخراج DNA با درجه خلوص بالا اهمیت زیادی در مطالعات مولکولی و موفقیت تکثیر مقدار ژن هدف دارد. هدف از انجام این مطالعه مقایسه روش‌های مختلف استخراج DNA در سوسک‌های پوست‌خوار به منظور انتخاب روشی مناسب با بالاترین کیفیت و کمیت است. نمونه‌برداری سوسک‌های پوست‌خوار از نقاط مختلف جنگل‌های شمال ایران انجام شد. ۵ روش مختلف استخراج DNA شامل چلکس، فنل کلروفرم، سی تب، نمکی و بافر استخراج بر روی نمونه‌ها در آزمایشگاه انجام و مورد ارزیابی قرار گرفت. کمیت و کیفیت DNA استخراج شده توسط دستگاه اسپکتوفتومتر و ژل الکتروفورز اندازه‌گیری شد. براساس نتایج بدست آمده میانگین کمیت و کیفیت DNA بدست آمده در روش‌های مورد مطالعه به ترتیب $23/6 - 579/7$ نانوگرم بر میکرولیتر و بالاترین جذب در $280/260$ ($1/8 - 0/9$) است. نتایج آنالیزهای آماری که توسط نرم افزار SPSS انجام شد تفاوت معنی‌داری را بین روش‌های مختلف استخراج DNA نشان می‌دهد. نتایج این مطالعه دو روش چلکس و نمکی را به عنوان مناسب‌ترین روش‌ها برای استخراج DNA سوسک‌های پوست‌خوار معرفی می‌کند.

واژگان کلیدی: سوسک‌های پوست‌خوار، استخراج DNA، جنگل، اسپکتوفتومتر