DNA recovery from microhymenoptera using six non-destructive methodologies with considerations for subsequent preparation of museum slides

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DNA recovery from microhymenoptera using six non-destructive methodologies with considerations for subsequent preparation of museum slides

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Abstract: Due to the tiny size of microhymenoptera, successful morphological identification typically requires specific mounting protocols that require time, skills and experience. Molecular taxonomic identification is an alternative, but many DNA extraction protocols call for maceration of the whole specimen, which is not compatible with preserving museum vouchers. Thus, non-destructive DNA isolation methods are attractive alternatives for obtaining DNA without damaging sample individuals. However, their performance needs to be assessed in microhymenopterans. We evaluated six non-destructive methods: A) DNeasy[®] Blood & Tissue, B) DNeasy[®] modified, C) Protocol with CaCl₂ buffer, D) CaCl₂ modified, E) HotSHOT modified and F) Direct PCR kit. The performance of each DNA extraction method was tested across several microhymenopteran species by attempting to amplify the mitochondrial gene COI from insect specimens of varying ages: 1 day, 4 months, 3 years, 12 years and 23 years.

Methods B and D allowed COI amplification in all insects, while techniques A, C and E were successful in DNA amplification from insects up to 12 years old. The technique F, the fastest, was useful in insects up to 4 months-old. Finally, we adapted permanent slide preparation in Canada balsam for every technique. The results reported allow for combining morphological and molecular methodologies for taxonomic studies.

Key words: Parasitic Hymenoptera, COI, museum insects, DNA extraction

Résumé: En raison de la petite taille des micro-hyménoptères, une identification morphologique réussie requière généralement des protocoles de montage spécifiques qui nécessitent du temps, des compétences et de l'expérience. L'identification taxonomique moléculaire est une alternative, mais de nombreux protocoles d'extraction d'ADN appellent à la macération de l'insecte en entier, ce qui est non compatible avec la conservation des spécimens de référence. Par conséquent, les méthodes d'isolement de l'ADN non destructives sont des alternatives intéressantes à l'obtention de l'ADN, permettant de conserver intact les individus de l'échantillon. Cependant, leur efficacité doit être évaluée sur les micro-hyménoptères. Nous avons évalué six méthodes non destructives: A) DNeasy® Blood & Tissue, B) DNeasy® modifié, C) Protocole de CaCl_2 , D) CaCl_2 modifié, E) HotSHOT modifié et F) Kit de PCR direct. L'efficacité de chaque méthode d'extraction d'ADN a été testée sur plusieurs espèces de micro-hyménoptère en essayant d'amplifier le gène mitochondrial COI à partir de spécimens d'insectes de différents âges: 1 jour, 4 mois, 3 ans, 12 ans et 23 ans.

Les méthodes B et D ont permis l'amplification du gène COI pour tous les insectes, tandis que les méthodes A, C et E ont permis l'amplification des insectes présentant jusqu'à 12 ans d'âge en conservation. La technique F, plus rapide, a été utile pour les insectes présents depuis 4 mois en conservation. Enfin, nous avons adapté le montage permanent de lames dans le baume du Canada pour chaque technique. Les résultats présentés permettent de combiner des méthodes morphologiques et moléculaires pour les études taxonomiques.

Introduction

The majority of the molecular techniques developed for extracting DNA from insects (and other organisms) require full or partial destruction of specimens resulting in the loss of morphological information (Favret 2005; Castalanelli et al. 2010). This is problematic if it is not possible to completely macerate a sample (or the required portion of it), for example, because the specimens belong to scientific collections or are unique or “type” specimens that serve as taxonomic references. This is particularly relevant in the case of samples that are to be included in the global bioidentification system known as the Barcode of Life Database, which requires adding genetic information, a voucher specimen, photographs and morphological classification (Hebert et al. 2003). To overcome this, researchers have developed semi-destructive and non-destructive DNA extraction techniques, in which the insect’s abdomen is punctured (Favret 2005; Castalanelli et al. 2010), a body segment is removed (Smith 2005) or the specimens are fully immersed in extraction buffers (Gilbert et al. 2007; Porco et al. 2010; Tin et al. 2014). Sonication has also been used in non-destructive methods (Hunter et al. 2008). DNA extraction methodologies based on boiling-freezing process have also been shown to be effective in isolating bacterial DNA and protein (Bej et al. 1991; Abd-Elsalam et al. 2011) and improving DNA extraction yields in yeasts (Almeida et al. 2012; De Baere et al. 2002).

The techniques of DNA extraction mentioned above have been developed in groups of non-insect arthropods (i.e., spiders and springtails) (Porco et al. 2010), or relatively large

insects like beetles (Gilbet et al. 2007; Thomsen et al. 2009), but not in taxonomic groups such as the microwasps. Microhymenoptera are often employed as biological control agents of insect pests, and some of them have been used worldwide in applied programs for the past 100 years. Representative specimens of these insects, such as the families Mymaridae, Trichogrammatidae, and Aphelinidae among others, can measure less than 0.5 mm in length (Gibson et al. 1997). In addition to their tiny size, museum specimens are delicate and usually require mounting on permanent slides into resin (Canada balsam). Unfortunately, there is no procedure for sequencing type specimens after mounting in resin, which makes it necessary to extract DNA before preparation of slides. Likewise, most published methodologies for preservation of microhymenopterans do not include recommendations for extraction or preservation of their DNA (Noyes 1982; Platner et al. 1999; Walker & Crosby 1988; Wirth & Marston 1968). There is also a lack of information about the effect of “storage” time or preservation method (alcohol, resins, natural drying and critical point drying) on DNA recovery. Therefore, advances in molecular and morphological identification must ideally be combined to capture the maximum amount of information.

The objective of this study was to evaluate six non-destructive DNA extraction methodologies and to determine whether it is possible to obtain complete sequences of COI in several species preserved for varying lengths of time; 1 day, 4 months, 3 years, 12 years and 23 years. In addition, the states of the post extraction insects were evaluated to

determine the effect of chemical solutions and temperatures on morphological characteristics. These morphological alterations should be considered when adapting permanent slide mounting techniques.

Materials and Methods

DNA Extraction

In order to determine the effectiveness and quality of DNA extraction processes, we selected five different microhymenopteran samples: two samples from different localities belonging to the family Trichogrammatidae (both *Trichogramma pretiosum*), two from the family Mymaridae (*Gonatocerus* and *Anagrus*) and one from the family Eulophidae (*Tamarixia radiata*). These hymenopterans represented different periods of conservation (i.e. 23 years, 12 years, 3 years, 4 months, 1 day) (Table 1). Each combination of a unique sample age and extraction method was performed in triplicate, and each methodology was evaluated according to the absence or presence of the expected DNA band in the PCR amplification, the intensity of this DNA band in the agarose gel electrophoresis.

DNA aliquots obtained from the youngest and oldest specimens from A, B, C, D, E and F extraction method were sequenced. The PCR products of the COI sequences were purified and sent for direct, Sanger sequencing to Genomic Services at CINVESTAV-

LANGEBIO (Irapuato, Guanajuato, Mexico). Sequences were aligned using Mega 6.06 software (Tamura et al. 2013) and published in GeneBank.

For all techniques, the amount of lysis buffer was adjusted according to the size of specimens, which ranged from 0.25 mm to 1 mm in length. Prior to DNA extraction, the specimens were preserved in ethanol (EtOH), and then dried for ten minutes on a filter paper. Dried specimens preserved in gelatin capsules were placed directly into the lysis buffer (Table 1). The methodologies are as follows:

Method A: DNeasy® Blood & Tissue (QIAGEN ©). The specimen was placed in 100 µL lysis buffer ATL, mixed with 20 µL of proteinase K, and incubated for 12 h at 55 °C. Subsequently, the mixture was transferred to a new 1.5 ml sterile tube. This mixture was used to continue total DNA purification using a spin-column protocol detailed by the manufacturer. Finally, the DNA was suspended in 20 µL of preheated TE buffer at 65 °C.

Method B: DNeasy® Blood & Tissue (QIAGEN ©), modified. We add a boiling-freezing process for improving DNA extraction yields. The same protocol as method A was used, but with the addition of an extra period of freezing at -20 °C for 8 h after the 12 h incubation at 55 °C.

Method C: Protocol with CaCl_2 buffer (Gilbert et al. 2007). Each specimen was completely immersed in 130 μl of lysis buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl, 3 mM CaCl_2 , 2% SDS (w/v), 40 mM DTT, and 0.25 mg/mL proteinase K), and incubated at 55°C for 16-20 h with an orbital shaker stirring at 35 rpm (MaxQ™ Thermofisher Scientific incubator). After digestion, the buffer was transferred to a new 1.5 ml tube. Subsequently, the nucleic acids were purified from the lysis buffer through extraction by phenol:chloroform, which was added to each sample in the same volume as the recovered lysis buffer and then mixed by vortexing. The mixture was then centrifuged for 10 min at 14,000 rpm and 22 °C. The supernatant was taken and placed in a new sterile 1.5 ml tube. Later, 0.6 and 0.1 volumes of cold isopropanol and cold ammonium acetate were added to the supernatant. The solution was gently mixed by inversion and centrifuged at 14,000 rpm for 25 min to form a DNA pellet. The supernatant was discarded, the pellet washed twice with 85% EtOH, and then centrifuged at 14,000 for 5 min. Finally, the pellet was dried with the open tubes facing up, and then it was resuspended in 40 μL of TE Buffer.

Method D: Protocol with CaCl_2 buffer (Gilbert et al. 2007), modified. We add a boiling-freezing process for improving DNA extraction yields. An extra 8 h freezing period at -20 °C was added after the cell lysis step of method C.

Method E. HotShot (Truett *et al.* 2000). A premixed lysis solution was prepared with 5 μ L of 100 mM NaOH and 15 μ L of 0.26 mM disodic EDTA per insect. The insect was immersed in 20 μ L of lysis solution and incubated for 30 min at 95 °C. Finally, 20 μ L of 40 mM Tris-HCl were added as a neutralizing solution. The final solution was transferred to a new sterile tube.

Method F: Direct PCR. The methodology followed used “Phire Tissue Direct PCR master mix (Thermo Scientific TM)”. The specimen was immersed in 20 μ L “Dilution buffer and storage solution” and 0.5 μ L “DNA release additive”, mixed by vortex and centrifuged briefly at room temperature. It was immediately incubated for 5 minutes at room temperature, and then it was vortexed, centrifuged and placed at 98 °C for 2 minutes. After incubation, it was centrifuged again and the supernatant was transferred to a new sterile tube.

At the end of each non-destructive DNA extraction, the nucleic acids were transferred to a new sterile tube and the insect was submerged into absolute ethanol.

PCR amplification. The extracted nucleic acids were amplified in a Veriti® 96-Well Thermal Cycler (Applied Biosystems), using primers for the gene Cytochrome C Oxidase I (COI), LCO1490 and HCO2198 (Folmer *et al.* 1994). Methods A, B, C, D and E for

mitochondrial DNA were performed in volumes of 25 μ L, including 2 μ L of DNA, 1X Green GoTaq® Flexi Buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 2 μ L BSA (10 mg/mL), 0.2 μ L to each primer (10 μ M), and 1 U GoTaq® G2 Flexi DNA Polymerase (Promega®). Cycling conditions were: 1 cycle at 94 °C for 30 s, 36 cycles of: 94 °C for 30 s, 50 °C for 45 s and 72 °C for 60 s, and one cycle at 72 °C for 5 min. PCR reactions of method F were developed in 20 μ L volumes containing 1X Phire Tissue direct PCR master mix (2X) that employs Phire Hot Start II DNA Polymerase (Thermo Scientific™), 0.4 μ L of each primer (10 μ M), nuclease-free water, and 2 μ L of DNA. Cycling consisted of one cycle of 98 °C for 5 min, 40 cycles at 98 °C for 5 s, 51 °C for 5 s and 72 °C for 20 s, and a final extension step at 72 °C for 5 min. Negative controls were included in all experiments.

PCR products were visualized by electrophoresis in a 1% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, USA) to confirm amplification of samples, intensity of bands, the presence or absence of non-specific products, and non-amplification of a no template control (NTC).

Slide mount. In microhymenopterans, most small specimens need to be mounted on a slide to be identified. The slide preparation process for each specimen began with a clearing step in 10% KOH. Clearing time depends on how dark the insect is. Each specimen was cleared until its color did not interfere with the visibility of the surface, sutures, seta, or those

morphological characteristics of the specific keys for each group. For example, the clearing step took three hours at 25 degrees for *Trichogramma* and *Anagrus* or three days for *Tamarixia*. This was followed by 5 min in acetic acid, a dehydration step with a series of ethanol solutions of ascending concentration (30%, 50%, 70%, 96%) water, and an acclimatization period in clove oil for 24 h. Finally, insects were placed on the slide, in a dorsal position, on a drop of 80 % Canada balsam diluted with clove oil. They were then incubated at 40 °C for 48 h. After that period, another drop of Canada balsam was added, and the cover glass was put in place (Noyes 1982; Platner et al. 1999). Slide preparations of each specimen were compared with control insects (e.g. insects of the same sample that were not used in any extraction method) under a microscope.

Specimens sufficiently clarified after DNA extraction were transferred directly to clove oil, without clearing with KOH or dehydration by ethanol. Finally, the specimens were mounted with Canada balsam as described above and analyzed under a microscope.

Results

DNA extraction

A summary of the extraction methods, as assessed by the presence of the COI-PCR amplified bands (~ 680 bp) is shown in Figure 1. Numbers of samples with positive PCR products of each extraction are shown in Table 2.

The intensity of the band in the agarose gel is associated with time in preservation. In newly collected insects (1 day) very bright bands were observed, whereas in older insects (23 years) the band intensity decreased or was absent (Fig. 1).

DNA extraction was possible in all methods, with some important variations. Methods B and D amplified the genetic material of all microhymenopterans. These methods differ from the methods A and C by an additional period of freezing. Regarding the DNA extraction from old samples (23 years old), it is important to note that the amplification was possible due to the addition of a freezing period to methods A and C, however the intensity of the bands was relatively fainter in agarose gel in comparison to the bands from more recently collected specimens (Figure 1). It was possible to successfully extract DNA with all methods in insects collected under 12 years, except for method F.

The estimated times for each non-destructive DNA extraction method are listed in Table 2. The most time consuming methods were A, B, C and D, which took 14 to 26 h, while methods E and F could easily be completed in less than an hour.

DNA sequences

COI nucleotide sequences were obtained from methodologies A, B, C, D, E, and F. DNA sequences generated with these methods, for the youngest (1 day-old) specimen of *T. radiata*, can be found in GenBank under accession numbers KU702643, KX501122, KU702645, KX501123, KU702647 and KU702649 respectively. However, the nucleotide readings from B and D for *T. radiata* were partial sequences. DNA sequences generated with methods A, C, E, for the oldest (12 years-old) specimen and F (4 months-old), from *T. pretiosum*, can be found in GenBank under accession numbers KU702640, KU702644, KU702646 and KU702648 respectively. Finally, a DNA sequence with very poor quality was obtained from the oldest specimen (23 years-old) *Gonatocerus* sp. with B and D methods, and no DNA sequence could be retrieved.

Slide preparation

All insects were examined under a microscope and were recovered intact (antennae, head, thorax, wings and abdomen still joined together) after DNA extraction. The A, B, C, D and E methods produce an exoskeleton discoloration in the smaller species such as *T. pretiosum* and *Anagrus*. There are also small external changes in relatively large insects such as *Gonatocerus* and *Tamarixia*. Methods C and D also caused a certain rigidity in all specimens, especially in the wings, which made them difficult to handle and set in the

optimal dorsal position on the slide. Only method F kept the characteristics and morphological structures intact in all used wasps.

Discussion

In recently collected insects, from one day to four months, we obtained high quality DNA with 6 non-destructive extraction methods. The release of DNA may be happening through mouth, anus, spiracles, and possibly through some areas of thin cuticle (Gilbert et al. 2007). Even though the DNA molecule is chemically stable, it does not preserve well under all storage conditions (Lindahl 1993). Results clearly show that, when isolating DNA, it is best to use recently collected insects. However, we found that it is possible to work with museum specimens up to 12 years old when preservation was done properly, preferably in 70 to 100% ethanol as suggested by Mandrioli (2008). The hymenopterans under study were preserved for varying lengths of time, under varying conditions and using different preservation techniques, but all specimens were preserved in highly concentrated ethanol solution (even *Gonatocerus*) and were dried after one month of being collected in the field. Unfortunately, dry museum specimens produced a thinner and fainter PCR band (Fig. 1), which was indicative of a low number of COI amplified copies due to degradation of DNA in the post-mortem storage conditions of these insects, possibly as a function of hydrolytic and oxidative decomposition, as proposed by Lindahl (1993). Consequently, the probability to obtain long (i.e. >600 bp) PCR amplicons is much lower for museum

specimens by effect of long-term preservation, which result in DNA of low quality for sequencing.

Only methods B and D, with an additional freezing step, show amplicons of COI region (Folmer et al. 1994) from 23 years old specimens, even though recovering a full length DNA sequence was not possible. We suggest trying the “mini-barcode” sequence to overcome these problems, because it proved useful in the sequencing of DNA from museum samples (Shokrolla et al. 2011; Lis et al. 2012; Meusnier et al. 2008). Another possibility is trying next generation sequencing methods such as RAD-tag (Tin et al. 2014) or targeted sequencing (Prosser et al. 2016), which allowed the sequencing of degraded DNA. It is a fact that the success in obtaining full-length barcodes from old museum specimens (i.e. > 10 years-old dried pinned insects) is almost always significantly lower than that from fresh samples of the same species (Shokrolla et al. 2011). It has been noted that during the cycles of freezing and thawing the cells are subject to cold osmotic shock and cell membrane ruptures (Moussa et al. 2008), which may facilitate the diffusion of DNA molecules out of the cell compartments. According to Almeida et al. (2012) freeze-thaw treatments are more injurious to the membrane of the yeast cells than boiling treatments and they could explain the advantage of this procedure over boiling for PCR amplification for samples with less time of preservation.

On the other hand, faint PCR bands, could be the result of DNA degradation, since DNA can be affected by storage conditions such as temperature, pH, exposure to UV radiation, or may have been degraded simply due to age. These factors are important for successful DNA sequencing; hence, when choosing a preservation method, it is important to consider such conditions to prevent enzymatic, chemical or physical breakdown of the nucleic acids (Lindahl 1993; Mandrioli 2008; Quicke et al. 1999).

Another important factor to consider when choosing a methodology for DNA extraction is the time required to process the samples. Despite being effective, methodologies A, B, C and D took a much longer time, (14-26 hrs.) (Table 2), whereas methodologies E and F produced PCR products in approximately 20 and 40 minutes, respectively.

Beside the differences in each DNA extraction method, we also found differences in the final specimens' morphological state, such as discoloration of the exoskeleton, transparency of the internal contents or stiffness in the specimens in comparison to the negative control. It is likely that the chemicals in A, B, C, and D methods caused this exoskeleton discoloration. These methods are more tedious and labor intensive than procedures E and F, which require soaking the specimen in buffer and a subsequent incubation.

Exoskeleton discoloration due to DNA extraction represents an advantage in the subsequent process of mounting the insects in slides, as in *T. pretiosum* and *Anagrus*. Methodologies A and B reduce the time for slide preparations, because after extraction the insects' cuticle and internal tissues are both clarified. Therefore, after the extraction of DNA the insect can be directly immersed in clove oil, eliminating the discoloration and dehydration steps (Fig. 2). In contrast, methodologies C and D do not reduce time in the slide preparation even when they clarify the exoskeleton, since they do not clear the insect internally (Fig 3). After using these methods it is necessary to place the specimens in KOH 10% for about six hours and follow all the steps of the standard process. When using these two techniques the specimens also become stiff, making it difficult to place the body flat on the slide and to spread the wings open. Methods E and F are the most suitable when preserving the wasps by mounting on card points, because they do not change the color of the exoskeleton.

In summary, the vast numbers of species to be barcoded require the use of specific methods to allow the morphological examination of voucher specimens, which is a key requirement for DNA barcoding and classical workflows. The six methods described here can be used in microwasps, but we conclude that the researcher must choose which is the adequate method for his/her investigation. The best decision will depend on how old the biological materials are, specimen size, and whether the specimens will be returned to a

scientific collection on card points or preserved on permanent slides for identification. It is important to note that the affinity of the primers used in the study may be different across species and that molecular evolution may have affected the gene sequence targeted by the primers. This is an issue that should be addressed in further investigations. In this paper we addressed the issue of isolating nucleic acids without destroying the specimens; however, the affinity of the primers on aging DNA was not tested, nor was the molecular changes along specimen age in any particular genera. In terms of time, only E and F methods were found to have great advantages. In the future, it will be convenient to test other methods for non-destructive DNA extraction from microwasps (Favret 2005; Hunter et al 2008), or to design new ones, which may be more efficient and cost effective.

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Table 1. Historical museum specimens used in this study

Species	Locality and collection dates (day.month.year)	Method	Preservation
1. <i>Gonatocerus</i>	Querétaro 15-VIII-1992	Sweeping net	Alcohol 96% and CPD*
2. <i>Trichogramma pretiosum</i>	Zapotlanejo, Jalisco. 27-III-2003	Direct-mass rearing	Alcohol 96%
3. <i>Anargus</i>	Saltillo, Coahuila. 29-I-2012	Moericke trap	Alcohol 96%
4. <i>Trichogramma pretiosum</i>	Chihuahua, Chihuahua. 06-XI-2014	Direct-mass rearing	Alcohol 96%
5. <i>Tamarixia radiata</i>	Tecomán, Colima. 24-II-2015	Direct-mass rearing	Alcohol 96%

* Critical point dry.

Table 2. Number of samples with positive PCR products from each extraction method and time required. (positive:number of repetitions).

Extraction Methods	Microhymenopterans species					Extraction time process
	1	2	3	4	5	
Method A	0:3	2:3	3:3	3:3	3:3	14 h
Method B	2:3	2:3	3:3	3:3	3:3	22 h
Method C	0:3	2:3	3:3	2:3	3:3	18 h
Method D	2:3	1:3	3:3	3:3	3:3	26 h
Method E	0:3	3:3	2:3	3:3	3:3	40 min
Method F	0:3	0:3	0:3	3:3	3:3	20 min

Method A. DNeasy® Blood &Tissue (QIAGEN®), Method B. DNeasy® Blood &Tissue (QIAGEN®), modified, Method C. Buffer CaCl₂ (Gilbert *et al.* 2007), Method D. Buffer CaCl₂ (Gilbert *et al.* 2007), modified, Method E. HotShot (Truett *et al.* 2000), Method F. Direct PCR. 1. *Gonatocerus* (15.VIII.1992), 2. *Trichogramma pretiosum* (27.III.2003), 3. *Anagrus* (29.I.2012), 4. *Trichogramma pretiosum* (06.XI.2014), 5. *Tamarixia radiata* (24.II.2015).

Fig. 1. PCR products of: A. DNeasy[®] Blood & Tissue Kit (QIAGEN[®], 2013); B. Method DNeasy[®] Blood & Tissue Kit (QIAGEN[®], 2013), modified; C. Protocol with CaCl₂ buffer (Gilbert *et al.*, 2007); D. Method with CaCl₂ buffer (Gilbert *et al.*, 2007), modified; E. HotSHOT method (Truett *et al.*, 2000); F. Phire Tissue Direct PCR master mix method (Thermo Scientific[™]). Samples: 1. *Gonatocerus* (15.VIII.1992), 2. *Trichogramma pretiosum* (27.III.2003), 3. *Anagrus* (29.I.2012), 4. *Trichogramma pretiosum* (06.XI.2014), 5. *Tamarixia radiata* (24.II.2015).

Fig. 2. Canada balsam slide (abbreviate the processes) of genital capsule of *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) representative of A and B method.

Fig 3. Canada balsam slide (abbreviate the processes) of genital capsule of *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) representative of C and D method.

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