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(Citation)
Applied Entomology and Zoology,52(2):349-352

(Issue Date)
2017-02-17

(Resource Type)
journal article

(Version)
Accepted Manuscript

Appl Entomol Zool (2017) 52:349–352
DOI 10.1007/s13355-017-0481-4

TECHNICAL NOTE

Evaluation of easy, non-destructive methods of DNA extraction from minute insects

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Abstract Although it is important to identify species of pest and natural enemy insects in agriculture, morphological identification alone is often difficult. Because a combination of morphological identification and DNA barcoding is very important for identifying indiscriminable species, there is a need to develop non-destructive DNA extraction methods. Here, we compared three general methods of DNA extraction—the Chelex method, PrepMan® Ultra Reagent, and the DNeasy® Blood and Tissue Kit— in the search for a rapid, simple, and non-destructive method of DNA extraction from pest and natural enemy specimens. Amplification of PCR production was achieved with the Chelex method and the DNeasy® Blood and Tissue Kit. However, not all specimens tested by the PrepMan® Ultra Reagent method yielded amplification products. Observation of the morphological features of specimens dried of *Encarsia formosa* revealed that all heads and leg segments remained intact after DNA extraction by each of the three methods. Almost all samples treated with PrepMan® Ultra Reagent method had large, discernible morphological changes, in particular, of the antennae and forewings, which are important features for identifying minute wasps. In contrast, the antennae and forewings of specimens on which we had used the Chelex method suffered little damage. Use of the DNeasy® Blood and Tissue Kit method did not alter the forewing features, but strong wrinkles appeared in the antennae. We discuss the DNA extraction techniques from the perspective of these results.

Keywords Chelex method · *Encarsia Formosa* · minute insects · non-destructive DNA extraction

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Introduction

Understanding the taxonomy of agricultural pests and their natural enemies is important for developing effective control and management programs (Parrella and Keil 1984; Rossman and Miller 1996). Identification of pests and their natural enemies typically uses morphological characteristics and is sometimes problematic, potentially failing to recognize a serious pest or causing alarm over a nonthreatening species (Hebert et al. 2003). Difficulties with identification may result from natural phenotypic variation within a species, the involvement of morphologically cryptic taxa, limitations of morphological keys, the need to identify partial specimens that lack important taxonomic characteristics, and a shortage of the experienced taxonomists required for identification (Hebert et al. 2003). A combination of morphological identification and DNA barcoding is being developed to overcome such problems (Floyd et al. 2009; Moritz and Cicero 2004).

Methods used to prepare insect samples for morphological identification often prevent the analysis of DNA from the specimen, whereas the column-based DNA extraction methods most frequently used on insects require maceration of the sample, destroying the morphological characteristics required for identification. For this reason, DNA extraction that may cause damage or loss of specimens cannot be performed on type specimens held in insect collections. Several non-destructive DNA extraction methods have been published; these allow the specimen to be identified by using DNA analysis without any obvious alterations to the morphological characteristics (Favret 2005; Gilbert et al. 2007; Hunter et al. 2008; Pons 2006; Rowley et al. 2007).

However, these methods require the use of toxic or corrosive chemicals (e.g. phenol, chloroform, and guanidine isothiocyanate); they can be time-consuming because of the need for overnight incubation, and they can risk the loss of DNA through ethanol or isopropanol precipitation. In addition, the deterioration of morphological characteristics after such DNA extraction has never been evaluated.

Our goal here was to develop a method of minimizing the damage caused by DNA extraction. We examined whether it was possible to conduct DNA extraction nondestructively by using well-known methods, namely the Chelex method which has been developed for studying extremely small organisms (Asghar et al. 2015; Barstead et al. 1991; Williams et al. 1992), an Applied Biosystems (ABI) product used to extract DNA safely and easily, and a QIAGEN kit. After DNA extraction, we evaluated the degree of deterioration of the form and essence of the specimen. We thus evaluated a method of extracting DNA without causing visible, external morphological damage to specimens.

Material and methods

Insects used

We used individuals of *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) sold by Agrisect Inc (Ibaraki, Japan). This wasp was chosen because it is minute, its morphological characteristics deteriorate easily, and it was easily available at the tie.

DNA Extraction, PCR, and DNA Sequencing

We used three DNA extraction methods, namely the Chelex method, PrepMan® Ultra Reagent (Applied Biosystems, Tokyo, Japan), and DNeasy® Blood and Tissue Kit (Qiagen, Tokyo, Japan). Twelve females were treated by using each method. With the Chelex method, DNA was extracted from individual specimens by uncrushing them singly in 30 µL of 5% Chelex® 100 Resin (143–2832 BioRad) of ultrapure water. The ultrapure water sterilized by autoclave was used. Then incubating them with 2 µL of 0.5 mg/mL proteinase K at 56 °C for 24 h. The specimens were boiled at 99.9 °C for 3 min to inactivate the proteinase K, and they were then used as templates for PCR. With the DNeasy Blood and Tissue Kit, we extracted DNA from specimens in

accordance with the manufacturer's protocol. Modifications to this protocol were as follows: (1) tissue was digested with proteinase K overnight at 37 °C; and (2) we used a final elution volume of 20 µL to increase the final DNA concentration of the eluate. With the PrepMan® Ultra Reagent we followed the following manufacturer's protocol.

PCR amplifications were conducted under the following conditions: 16.5 µL of AmpliTaq Gold PCR Master Mix (PE Applied Biosystems, Tokyo, Japan), 1.3 µL of forward and reverse primer (10 pmol/µL) and 13.9 mL of sterile water, to give a total of 33µL PCR reaction volume. The COI region was amplified by using LCO1490 (5´-GGT CAA CAA ATC ATA AAG ATA TTG G-3´) and HCO 2198 (5´-TAA ACT TCA GGG TGA CCA AAA AAT CA-3´) primers (in accordance with the method of Folmer et al. 1994). PCR amplifications were done in an ABI thermal cycler (PCR System 9700, Applied Biosystems) with the following program: initial denaturing at 92 °C for 1 min; 35 cycles of 92 °C for 1 min, annealing at 58 °C for 1 min, and 72 °C for 1 min 30 s; and a final extension step of 72 °C for 1 min 30 s. All PCRs included a negative control (sterile water instead of DNA) to detect DNA contamination. PCR products were resolved on 1.5% agarose gels, stained with ethidium bromide, and visualized under a UV transilluminator.

Statistical analyses were performed with R version 2.15.0 (R Development Core Team 2012). Percentage detections of bands in PCR using each method were compared by using Fisher's exact test with Tukey's multiple comparisons.

PCR products were sequenced by using the direct sequencing method. A dye-terminator-labeled cycle sequencing reaction was conducted with a BigDye DNA Sequencing Kit ver. 3.0 (PE Applied Biosystems). Reaction products were analyzed with an ABI PRISM 3137 Genetic Analyzer (PE Applied Biosystems). The partial sequences were edited and connected by using Sample Manager (PE Applied Biosystems). Sequence waves were estimated on a scale of 0 or 1, where 0 = unreadable and 1 = readable.

Drying specimens removed from DNA extraction buffer

Specimens from which DNA had been extracted were dried with

hexamethyldisilazane (HMDS). A number of methods are used to dry minute wasps picked up liquids (Noyes 2010), but we used HMDS here because of its relatively good results and better accessibility than with the use of a critical point drier or freeze drier. The methods listed below mainly followed those of Heraty and Hawks (1988) and Noyes (2010).

1) Transfer each specimen from the DNA extraction buffer to 70% EtOH (in a glass tube), then leave at least 10 min; 2)pour out the 70% EtOH and add 80% EtOH, then leave for at least 10 min; 3) pour out the 80% EtOH and add 90% EtOH, then leave for at least 10 min; 4) pour out the 90% EtOH and add 99% EtOH, then leave for at least 10 min; 5) pour out the 99% EtOH and add HMDS, then leave for at least 30 min; 6) transfer each specimen to HMDS in a ceramic dish (with a cover), then leave for at least 30 min; and 7) pour out most of the HMDS, then allow the remaining HMDS to evaporate gently from the specimen in a large box.

Morphological examination

Specimens from which DNA had been extracted by using the three methods were mounted on card points with water soluble glue. The morphological features of the mounted specimens were examined under a binocular stereomicroscope (Nikon SMZ1000). Morphological features were estimated on the antennae and forewings (a rank of 0 to 3, where 0 = bad, 1 = moderate to bad, 2 = moderate, and 3 = good), head (a rank of 0 to 2, where 0 = bad, 1 = moderate, and 2 = good), and metasoma and legs (a rank of 0 or 1, where 0 = bad and 1 = good).

The morphological index for each part was calculated by using the formula:

Morphological index = ($\Sigma i \times ni$) / (maximum morphological features $\times$ N), where

N, total number of specimens examined; ni, number of specimens of each rank; i, number of specimens classified into category i.

Statistical analyses were performed with R version 2.15.0 (R Development Core Team 2012). The morphological index values for each part were analyzed by using analysis of variance (ANOVA) with Tukey's multiple comparisons.

Results and discussion

Table 1 summarizes the results of amplification of the targeted genes using each method of DNA extraction. Generally, with the pair of primers, we

obtained successful amplification of products from 600 to 700 bp. The percentage detection of bands with the Chelex method was significantly higher than with the PrepMan® Ultra Reagent method ($P < 0.01$, Fisher's exact test). There were no significant differences in the percentage detection of bands between the Chelex method and the DNeasy® Blood and Tissue Kit ($P > 0.05$, Fisher's exact test). The waves of sequences were readable with the Chelex method and the DNeasy® Blood and Tissue Kit, but not with the PrepMan® Ultra Reagent method.

Figure 1 shows the morphological features of pecimens dried of *E. formosa* after use of the three DNA extraction methods, along with those of a control treated only with HMDS.

Heads and leg segments were all intact after the application of each of the three methods.

All samples treated with PrepMan® Ultra Reagent method showed large, discernible morphological changes. The antennae and forewings are important features for the identification of minute wasps. The antennae of nine individuals were absent or heavily destroyed, and there were strong wrinkles in the forewings of 11 individuals. Several specimens were observed to have a translucent material attached to them; this was probably residue of the reagent (Fig. 1b).

In contrast, with the Chelex method, the antennae and forewing features of the specimens showed few effects of treatment (Table 2). Similarly, the DNeasy® Blood and Tissue Kit method did not alter the features of the forewing, although there was strong wrinkling of the antennae.

The Market price of Chelex® 100 Resin is 0.032 JYN per individual. In contrast, the market price of DNeasy® Blood and Tissue Kit (50) is 460 JYN per individual.

Many previously described methods of DNA extraction from arthropods require the use of multiple steps, toxic or corrosive chemicals, or expensive components (Favret 2005; Gilbert et al. 2007; Hunter et al. 2008; Pons 2006;

Rowley et al. 2007). In contrast, the Chelex method is inexpensive, time and labor efficient, and of low toxicity, allowing the extraction of DNA for amplification without damage to the external characteristics. But in the future, we would have to examine relationship between extracted DNA concentration and processing time by the Chelex method.

As a non-destructive DNA extraction method, the Chelex method was very useful and would merit being used more frequently, especially in the case of tiny insects for which the whole body is needed for DNA extraction. But it would require attention because the situation may be different in all insects. The Chelex method allows the detection of any initial misidentifications. Furthermore, it enables the vouchering of barcoded specimens (Hunter et al. 2008; Rowley et al. 2007), which in turn potentially decreases the chance of Type I identification errors.

Acknowledgments

This research was in part supported by JSPS KAKENHI Grant Number 25292034 to K. Maeto and K. Miura.

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Figures legends

Figure 1 Morphological features of mounted specimens extracted by using three methods, (a) Chelex method, (b) Prepman® Ultra Reagent, (c) DNeasy Blood and Tissue Kit and (d) control.

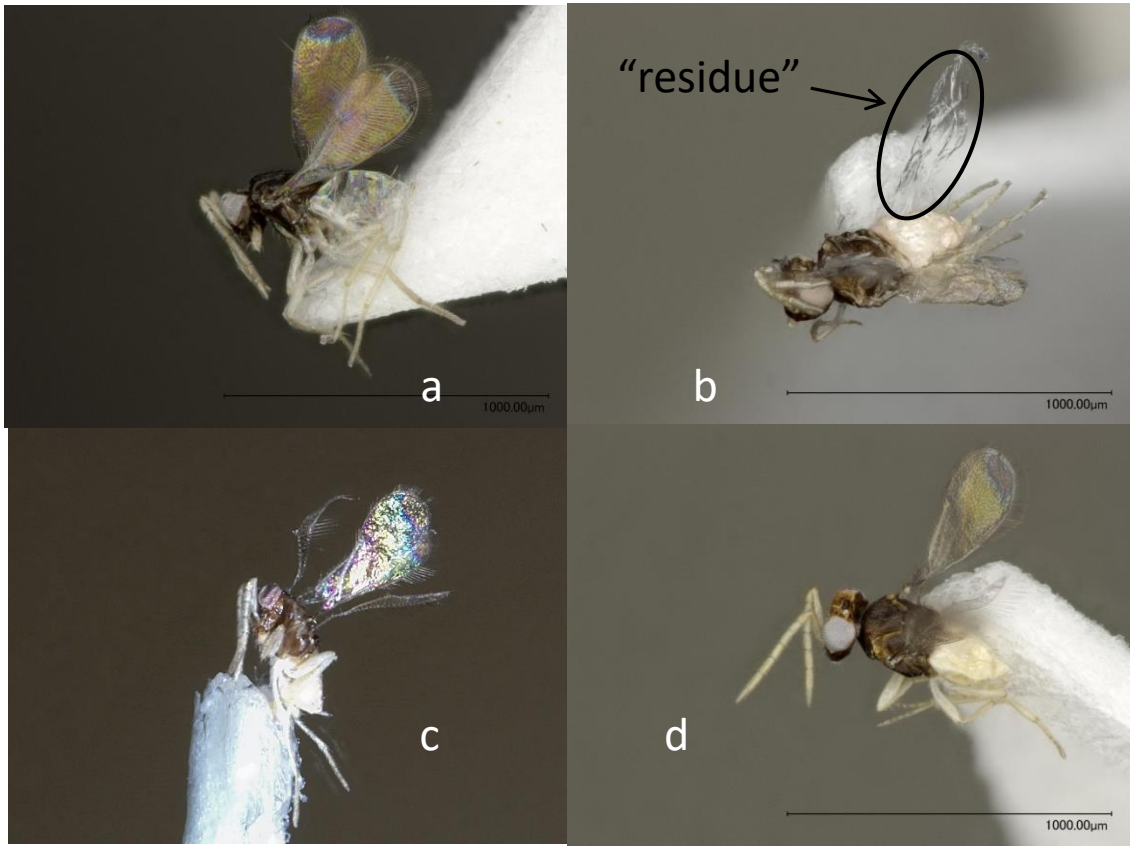

Figure 1 Morphological features of mounted specimens extracted by using three methods, (a) Chelex method, (b) Prepman® Ultra Reagent, (c) DNeasy Blood and Tissue Kit and (d) control.

Table 1 Summary of results pf amplification of targeted genes in three method,
Chelex method, PrepMan® Ultra Reagent and DNeasy® Blood and Tissue Kit, for DNA extraction

Methods	No of females treated	% detection of band in PCR	Index of condition in sequence
Chelex	12	100 a*	1
PrepMan® Ultra Reagent	12	25 b	0
DNeasy® Blood and Tissue Kit	12	66.7 ab	1

*Different letters in the same column indicate significant differences by Fisher's exact test with Tukey's multiple comparisons ($p < 0.05$)

Table 2 Morphological index of mounted specimens extracted by using three methods, Chelex method, PrepMan® Ultra Reagent and DNeasy® Blood and Tissue Kit.

Methods	Anntenae		Head		Forewing		Abdomen		Leg	
Chelex	0.72	a*	0.73	a	0.76	a	0.00	a	1.00	a
PrepMan	0.39	c	0.92	ab	0.53	b	1.00	b	1.00	a
Qiagen	0.44	c	0.54	ab	0.97	a	1.00	b	1.00	a
Control**	1.00	b	0.96	b	0.97	a	1.00	b	1.00	a

* Different letters in the same row indicate significant differences by Tukey's multiple comparisons after ANOVA ($p < 0.05$)

**Handled by only HMDS