Effects of Different Pretreatments of DNA Extraction from Dried Specimens of Ladybird Beetles (Coleoptera: Coccinellidae)

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Abstract: Obtaining genetic information from museum specimens is a fundamental component of many fields of research, including DNA barcoding, population genetics, conservation genetics, and phylogenetic analysis. However, acquiring genetic information from museum specimens is challenging because of the difficulty in amplifying the target sequences due to DNA damage and degradation. Different pretreatments can significantly impact the purity and concentration of genomic DNA from museum specimens. Here, we assessed four pretreatment methods—use of 0.9% NaCl buffer, phosphate-buffered saline (PBS), Saline Tris-EDTA (STE) buffer, and sterile water—to determine which pretreatment is most suitable for DNA extraction from dried specimens of ladybird beetles. We completed a comprehensive phylogenetic analysis to test whether the sequences obtained from dried specimens enable proper phylogenetic inference. Our results showed that pretreatment can improve the quality of DNA from dried specimens. The pretreatment effects of 0.9% NaCl buffer and STE buffer were better than those of PBS buffer and sterile water. The phylogenetic analyses results showed that museum specimens can be used to generate cogent phylogenetic inferences. We report the optimum pretreatment methods for DNA extraction from dried ladybird beetles specimens as well as provide evidence for accurately determining phylogenetic relationships for museum specimens.

Keywords: pretreatment; DNA extraction; dry specimens; phylogenetic analysis; Coccinellidae

1. Introduction

Developments in molecular biology, specimen identification, and phylogenetic and population genetics require the use of molecular techniques, and DNA sequences provide vast quantities of information for phylogenetic inference and taxonomic identification. However, obtaining and collecting fresh material is time-consuming, expensive, and often fails to provide a wide coverage of the species [1]. Museum specimens generally cover a broader taxonomic range and are more easily obtained, enabling a wider range of questions and taxa to be studied [2]. Many researchers are trying to exploit this potential of museum specimens [3–5] and some museums are becoming active molecular genetics research institutions. Museums worldwide house millions of animal and plant specimens, many of which have been preserved so that scientific investigation is possible. Hence,
museum specimens have become an important source of data in population genetics, conservation genetics and phylogenetic inference studies [2]. For research on museum specimens, high-quality DNA from dried specimens is required.

Advancements in molecular biology have enabled the extraction of genomic DNA from historic and even ancient tissue specimens [6]. However, evident limitations exist when using DNA from museum specimens; obtaining sufficient amounts of high-quality DNA is the main challenge. In general, not only age but also storage and preservation methods affect DNA quality and the amplification success [1]. Results of earlier studies indicated that different pretreatment methods can significantly impact the purity and concentration of DNA extracts from dried insect specimens [7–10].

Generally, many museum specimens, particularly dry-preserved insects, are stored pinned without any further preservation treatment [11]. Whereas the exoskeleton of the insects is stable for many years, the soft tissue soon dries out and decomposes [12]. Consequently, pretreatment is extremely important for DNA extraction from dried insect specimens. Previous studies reported a significant impact of pretreatment on the quality and purity of dried insect specimen DNA, favorable for polymerase chain reaction (PCR) success and other molecular techniques [7,8].

Phylogenetic studies are vital for addressing biological questions as about the relationships among species or genes, the origin and spread of species and the demographic changes and migration patterns of species [13]. Therefore, using museum specimens can help with forming correct phylogenetic inferences, also enabling a wider range of questions to be studied. In the present study, we assessed the effects of different kinds of DNA extraction pretreatment from dried specimen. We chose specimens of ladybird beetles (Coleoptera: Coccinellidae) for the current study as the species is highly abundant in the temperate zone, being important insect natural enemies and the subject of many ecological studies [14]. We amplified two mitochondrial genes: cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S) and one nuclear gene histone subunit 3 (H3) from dried ladybird beetles, and then applied four dominant phylogenetic analysis methods—neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI)—to test whether these sequences are an appropriate basis for phylogenetic inference. The objectives of this study were to (1) identify a best-practice approach for pretreatment of high-quality DNA extracts from dried specimens, and (2) test whether museum specimens can be used successfully for determining reproducible phylogenetic relationships.

2. Materials and Methods

2.1. Specimens

All specimens were obtained from the Engineering Research Center of Biological Control, Ministry of Education, South China Agriculture University (SCAU, Guangzhou, China). Dried pinned specimens of Afissula expansa (Dieke, 1947), Epilachna plicata Weise, 1889, Hippodamia variegata (Goeze, 1777) and Scymnus (Pullus) kawamura (Ohta, 1929) were selected. In total, we collected 20 samples, 5 of each species, and applied the 4 methods of pretreatment and a blank control (Table 1). Samples were checked if they were mildewed and vermiculated under a stereoscope, to ensure there was no cross-contamination of DNA from fungi or something else.

2.2. Pretreatments

The specimens each underwent one of the following methods: 0.9% NaCl buffer, Saline Tris-EDTA (STE) buffer (0.1 mol/L NaCl, 10 mmol/L Tris-Cl pH 8.0, 1 mmol/L ethylene diamine tetraacetic acid (EDTA) pH 8.0), phosphate-buffered saline (PBS) buffer, or sterile water. All specimens were placed in a reaction tube. After the respective pretreatment, buffer solution or sterile water was added until the specimens were submerged and steeped for 3 h at room temperature. Likewise, the blank control was placed in a reaction tube and allowed to sit for 3 h at room temperature without any further treatment.
Table 1. Information on sample pretreatments, collection date, species and code information of museum specimen of five different coccinellid species used in the experiment.

| Pretreatment        | Code | Collection Date | Species          | GenBank Accession |
|---------------------|------|-----------------|------------------|------------------|
| 0.9% NaCl buffer    | A1   | 2009            | Afissula expansa | MK138697 MK190427 MK190447 |
| PBS buffer          | A2   | 2009            | A. expansa       | MK138698 MK190428 MK190448 |
| STE buffer          | A3   | 2009            | A. expansa       | MK138699 MK190429 MK190449 |
| Sterile water       | A4   | 2009            | A. expansa       | MK190430 MK190450   |
| CK                  | A5   | 2009            | A. expansa       | MK190431 MK190451   |
| 0.9% NaCl buffer    | B1   | 2009            | Epilachna plicata| MK138700 MK190432 MK190452 |
| PBS buffer          | B2   | 2009            | E. plicata       | MK138701 MK190433 MK190453 |
| STE buffer          | B3   | 2009            | E. plicata       | MK190434 MK190454   |
| Sterile water       | B4   | 2009            | E. plicata       | MK138702 MK190435 MK190455 |
| CK                  | B5   | 2009            | E. plicata       | MK190436 MK190456   |
| 0.9% NaCl buffer    | C1   | 2008            | Hippodamia variegata | MK138705 MK190442 MK190461 |
| PBS buffer          | C2   | 2008            | H. variegata     | MK138703 MK190438 MK190458 |
| STE buffer          | C3   | 2008            | H. variegata     | MK138704 MK190439 MK190459 |
| Sterile water       | C4   | 2008            | H. variegata     | MK190440 MK190460   |
| CK                  | C5   | 2008            | H. variegata     | MK190441 -           |
| 0.9% NaCl buffer    | D1   | 2012            | Scymnus (Pullus) kawamurai | MK138705 MK190442 MK190461 |
| PBS buffer          | D2   | 2012            | S. (P) kawamurai | MK138706 MK190443 MK190462 |
| STE buffer          | D3   | 2012            | S. (P) kawamurai | MK138707 MK190444 MK190463 |
| Sterile water       | D4   | 2012            | S. (P) kawamurai | MK138708 MK190445 MK190464 |
| CK                  | D5   | 2012            | S. (P) kawamurai | MK138709 MK190446 MK190465 |

2.3. DNA Extraction and PCR Amplification

All samples were moved to a new reaction tube for total genomic DNA extraction following pretreatment. All DNA extractions were completed with the Qiagen DNA Blood and Tissue kit (TianGen Biochemistry, Beijing, China) following the protocol provided by the manufacturer. A spectrophotometer (NanodropTM, ThermoFisher Scientific, Waltham, MA, USA) was used to measure the OD$_{260/280}$ ratio for characterizing the DNA quality. An OD$_{260/280}$ value below 1.6 indicates that the DNA extract has been contaminated by protein or phenol, whereas an OD$_{260/280}$ value above 1.9 indicates that the DNA extract has been contaminated by RNA.

Two mitochondrial genes, cytochrome-c oxidase subunit I (COI) and 16S ribosomal RNA (16S), and one nuclear gene histone subunit 3 (H3), were amplified to assess the quality of the DNA extracts. PCR was conducted in 25 µL volumes including 12 µL SuperMix (TransGen Biotech, Beijing, China), 10 µL deionized H$_2$O, 1 µL template and 1 µL each of primer. PCR cycle conditions for the three genomic regions were similar: an initial denaturation of 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, and a final extension at 72 °C for 5 min. PCR products were electrophoresed on 1.0% agarose gel. DNA fragments were sequenced in both directions with sufficient overlap to ensure the accuracy of sequence data. Sequencing was performed by Sangon Biotech, Shanghai, China. Raw sequences were assembled and edited in Geneious 9.1.5 [17], manually checked for sequencing errors and ambiguities, and then BLASTed in GenBank. The detail information is shown in Table 2.

Table 2. Information on the primer sequences and corresponding genes information.

| Marker | Primer Name | Primer Sequence (5’-3’) | Reference |
|--------|-------------|-------------------------|-----------|
| COI    | Jerry       | CAACATTTATTTTGATTTTT    | [15]      |
|        | Spat        | GCACIAWTCGCTCATATTAGA   | [15]      |
| 16S    | 16S A       | CGCTCTTTATCAAAAACAT     | [16]      |
|        | 16S B       | CTCCGGTTGGAACCTCAGATCA  | [16]      |
| H3     | H3F-1       | CAGAAAGTCGACGGAGAGCGAAG | This study |
|        | H3R-1       | GCCGTTTGCGTGAGTGGCG     | This study |
2.4. Sequences Composition and Phylogenetic Analysis

The base composition and the number of parsimony informative sites were calculated using MEGA 7.0 [18]. Alignments of the individual makers were linked in SequenceMatrix [19]. Neighbor-Joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) approaches were applied to test whether these sequences enable correct phylogenetic inference. An NJ tree was constructed in MEGA 7.0 based on the Kimura-2-parameter (K2P) model using a combined dataset. *Endomychus biguttatus* Say (COI: GQ302304, 16S: GQ302094, and H3: GQ302447) and *Corynomalus vestitus* Voet (COI: GQ302321, 16S: GQ302115, and H3: GQ302462) were downloaded from GenBank as an outgroup to root the tree. These species belong to the Endomychidae. This family has been identified as a sister-group of the Coccinellidae within superfamily Coccinelloidea [20,21].

MP analyses were performed using PAUP 4.0 [22]. Heuristic searches were conducted using tree bisection reconnection (TBR) branch swapping, with 1000 random-addition replicates. ML and BI analyses were performed in RAxML 8.2.8 [23] and MRBAYES 3.2.6 [24]. Modeltest [25] was used to select an appropriate model of sequence evolution for each gene under the Akaike information criteria (AIC). PartitionFinder 1.1.1 [26] was used to find the best-fit substitution model for each partition based on the synthesized dataset. The analysis was run using all search schemes, with all models considered based on the AIC. ML was analyzed using the 1000 rapid bootstrapping replicates. For BI analyses, all model parameters were unlinked. Two MCMC runs were conducted with one cold chain and three-headed chains (temperature set to 0.1) for 20 million generations and sampled every 1000 generations. The first 25% of the total trees were discarded as ‘burn-in’ and the remaining trees were used to generate a majority-rule consensus tree. The chain stationarity was visualized by plotting likelihoods against the generation number using the program TRACER 1.6 [27].

3. Results

3.1. Measurement of DNA Purity and Concentration

DNA purity and concentration varied amongst the different pretreatments (Table 3). The OD<sub>260/280</sub> value was species specific. The OD<sub>260/280</sub> for four different pretreatment methods with *E. plicata* and *S. (P.) kawamurai* resulted in higher purity values whereas the OD<sub>260/280</sub> for *H. variegata* indicated that the DNA extracts may have been contaminated by RNA. The different pretreatment methods impacted DNA purity, especially for *E. plicata* and *S. (P.) kawamurai*. However, the OD<sub>260/280</sub> of the sterile water treatment was 2.003, which was greater than the value for the blank control (1.983) in *A. expansa*. Apart from DNA purity, different pretreatments impacted on DNA concentration. Here, the DNA concentration of *E. plicata*, *H. variegata*, and *S. (P.) kawamurai* was higher than those of the blank control.

3.2. Amplification of Sequences

In total, we obtained 58 DNA sequences, and all sequences were BLASTed in GenBank. All COI and H3 fragments were confirmed as the targeted genomic region whereas 16S had six sequences that were non-specifically amplified, A4, A5, B3, B5, C1 and C4, and non-specific sequences were removed. Finally, we obtained 52 targeted DNA sequences: 13 16S sequences, 20 COI sequences and 19 H3 sequences. The target bands of 16S, COI and H3 of *H. variegata* on the agarose gel were weak (Figure 1). The blank control of *H. variegata* C5 did not produce PCR product for 16S. Likewise, for H3 of *H. variegata*, the control resulted in no band whereas pretreatment samples displayed an amplified target region (Figure 2).
Table 3. DNA purity and concentration of the different pretreatments. The code in this table corresponds to the code in Table 1.

| Pretreatment          | Code | OD<sub>260/280</sub> | Concentration (ng/μL) |
|-----------------------|------|----------------------|------------------------|
| 0.9% NaCl buffer A1   | 1.936| 433.159              |
| B1                    | 1.798| 44.513               |
| C1                    | 2.075| 61.52                |
| D1                    | 1.899| 69.99                |
| PBS buffer A2         | 1.966| 539.647              |
| B2                    | 1.929| 202.086              |
| C2                    | 2.067| 34.331               |
| D2                    | 1.924| 20.108               |
| STE buffer A3         | 1.942| 464.019              |
| B3                    | 1.823| 93.246               |
| C3                    | 2.074| 31.527               |
| D3                    | 1.864| 26.774               |
| Sterile water A4      | 2.003| 678.726              |
| B4                    | 1.98 | 52.972               |
| C4                    | 2.071| 32.554               |
| D4                    | 1.947| 17.117               |
| CK A5                 | 1.983| 537.078              |
| B5                    | 2.007| 26.408               |
| C5                    | 2.088| 14.513               |
| D5                    | 2.090| 23.265               |

The edited and aligned sequences lengths for 16S, COI, and H3 are 523 bp, 867 bp, and 290 bp, respectively. The average contents of A, T, G, and C were 35.7%, 40.7%, 14.9%, and 8.7% for 16S; 30.6%, 38.1%, 14.7%, and 16.6% for COI; and 23.5%, 18.9%, 27.8%, and 29.7% for H3, respectively. The number of parsimony informative sites of 16S, COI, and H3 were 86, 230, and 68, respectively. After linking the three genes, our final aligned fragment length was 1680 bp, which contained 384 parsimony informative sites. The average contents of A, T, G, and C were 30.4%, 35.1%, 17.2%, and 17.3%, respectively.

3.3. Phylogenetic Analyses

PartitionFinder results showed that each gene was partitioned separately, and GTR + I + G as the most appropriate model for each gene. Four phylogenetic reconstruction methods (NJ, MP, ML, and BI) of linked data yielded similar topologies (Figure 3). Four main clades could be distinguished, all highly supported statistically. The five samples of the same taxa formed a clade. The AIC, as implemented in Modeltest, yielded the GTR + I + G model of sequence evolution as most appropriate for COI, 16S, and H3. The phylogenetic results based on each gene were the same as the results based on linked data (Figures S1–S3). Our phylogenetic analyses showed that these sequences obtained from dry specimens could be used for phylogenetic inference.
Figure 1. Polymerase chain reaction (PCR) amplification of three genes: (a) 16S, (b) COI, and (c) H3. The code in the agarose gel image corresponds to the code in Table 1.

Figure 2. Amplification efficiency of 16S, COI and H3. Three different shades of squares are used to represent the PCR and sequencing results. The code in this figure corresponds to the code in Table 1.
Figure 3. Cladogram derived from analyses of the three markers demonstrating the phylogenetic relationships based on neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) of the combined dataset (1680 bp) under partition strategies. The support rate of the branches from left to right represents bootstrap values for NJ analysis, bootstrap values for MP analysis, bootstrap values for ML analysis, and posterior probabilities for BI. The codes following the sample name in this figure correspond to the code in Table 1. The vacant part of the circle on the right side of the tree represents target genes that we failed to amplify or sequence.

4. Discussion

In the present study, we assessed the effects of four different pretreatment methods on the quality of DNA extracts of museum specimens including the testing of the amplification success using three different genomic regions. Our results indicate that different pretreatments can improve DNA purity
and concentration, and the 0.9% NaCl buffer and STE buffer produced the highest DNA purity and quality. Pu et al. [9] stated that genomic DNA could be successfully extracted from dried specimens of Hymenoptera, which were collected in 1980 and 1987 using STE buffer pretreatment [9]. An et al. [7] also used STE buffer as a pretreatment for DNA extraction of dried specimens of the tribe Platyopini (Coleoptera: Tenebrionidae), which were collected from 1987 to 2008. Their results showed that high-quality genomic DNA could be extracted and they successfully amplified DNA fragments in PCR. Li et al. [8] compared the pretreatment effects of 0.9% NaCl buffer and sterile water for DNA extraction of Prostephanus truncatus, Callosobruchus maculatus, and Sitophilus oryzae using different reaction times. Their results showed that bathing in 0.9% NaCl buffer for three hours was the best for DNA quality. In this study, the effects of pretreatments by PBS buffer was inferior to 0.9% NaCl buffer and STE buffer in A. expansa, E. plicata and S. (P.) kawamura, but not for H. variegata. Pretreatments employing PBS buffer have already been shown to increase the DNA quality of specimens stored in alcohol. Generally, PBS gradually restores cells to the original physiological status due to slow permeation and gradually promotion of the cross-linking protein separation of DNA [10]. Conversely, pretreatment with sterile water can lead to severe cell damage due to high water absorption, followed by a dispersion of the cellular contents. Subsequently, partial genomic DNA will be lost [8].

For COI, we achieved excellent amplification success, even though this is the longest sequence of the three molecular markers investigated. The nuclear gene H3 presented 19 target bands, whereas the mitochondrial 16S had a high PCR success rate, but six sequences presented non-specific amplification. A similar situation was observed for the PCR amplification of DNA from ancient samples [28]. Although mitochondrial DNA is more easily amplified from suboptimal DNA extracts than nuclear genes [29], damaged templates may cause incorrect bases to be incorporated in the PCR product [30]. Most errors involved C→T substitutions on the L-strand, presumably due to deamination of cytosine in the template [31]. For 16S, PCR and sequencing results indicated that the pretreatments seem to have a positive effect on the quality of the DNA extract in increasing the chance for amplification success, but this was not true for COI and H3.

Our phylogenetic analyses revealed four highly supported clades, and the same taxa clustered together (Figure 3). Within high-level phylogenetic analyses, both A. expansa and E. plicata were shown to belong to the tribe Epilachnini [32]. In our analyses, both A. expansa and E. plicata recovered a monophyly with very good support for their taxonomic status. As mentioned above, our analyses proved that museum specimens can be employed for cogent phylogenetic inference.

5. Conclusions

In this study, we assessed the effects of four pretreatment methods—bathing in 0.9% NaCl buffer, PBS buffer, STE buffer, and sterile water—to identify the best practice for high-quality DNA extractions using dried ladybird beetles specimens. Our results showed that pretreatment can improve the quality of DNA. The addition of 0.9% NaCl buffer and STE buffer had better effects than PBS buffer and sterile water. Comprehensive phylogenetic analyses showed that museum specimens can be accurately used for phylogenetic inference. Overall, we identified appropriate pretreatment methods for DNA extraction from dried specimens and provided evidence that museum specimens can be used to correctly determine phylogenetic relationships. In order to effectively identify the most appropriate pretreatment, more replicates per species and treatments are needed in our further studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/10/4/91/s1, Figure S1: Cladogram derived from analyses of the COI markers; Figure S2: Cladogram derived from analyses of the 16S markers; Figure S3: Cladogram derived from analyses of the H3 markers.

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