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TECHNIQUES AND METHODOLOGY

Room temperature DNA storage with slide-mounted aphid specimens

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Abstract. 1. Microscope slide-mounted insect specimens present special problems for DNA extraction due to chemical clearing techniques and subsequent specimen preservation.

2. Filter paper is routinely used for DNA storage in other biological disciplines (e.g. medicine), but is not widely used in entomology.

3. A system for room temperature storage of DNA with microscope slide-mounted voucher material uses filter paper in conjunction with the cleared voucher specimen.

4. The viability of filter paper for DNA storage is demonstrated through various time intervals. Either a solution of the cell lysis buffer or purified DNA can be stored on filter paper. Both COI and COII were sequenced from filter paper; EF-1α was attempted, but results were partially successful.

5. The filter paper system allows for DNA storage directly with the curated specimen, thus facilitating its accessibility.

6. The storage of DNA on filter paper with specimens in museum collections could reduce the need or cost for cryopreservation equipment.

7. DNA on filter paper facilitates international and local transport of DNA research material for subsequent study.

Key words. Barcode, COI, COII, DNA storage, filter paper, Sternorrhyncha.

Biological collections are vitally important as repositories of voucher specimens and as sources of historic specimens for molecular studies (Page *et al*., 2004; Suarez & Tsutsui, 2004; Allmon, 2005; Dosmann, 2006; Winston, 2007; Hackett, 2010). Some insect preservation techniques such as pinning and point mounting allow for subsequent DNA extraction (Gilbert *et al*., 2002; Junqueira *et al*., 2002; Goldstein & DeSalle, 2003; Zimmermann *et al*., 2008); however, in other preservation techniques, most notably slide mounting, DNA recovery may not be possible. Typical techniques for slide mounting insects usually include a step where the specimen is cleared using a caustic agent, such as KOH, chloral phenol, or Hoyer's medium, which degrades the DNA and removes soft tissue (Hille Ris Lambers, 1950; Wilkey, 1962; Wirth & Marston, 1968). Additionally, microscope slides are typically kept in low temperature ovens (ca. 45–55 °C) for extended periods of time to cure the mounting medium, further degrading any DNA.

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For DNA extractions from insects that require slide mounting, fresh specimens or specimens freshly preserved in alcohol are needed. Frequently, these specimens are destroyed in the extraction process (e.g. Moran *et al*., 1999; von Dohlen *et al*., 2006; Foottit *et al*., 2009) and the absence of a voucher specimen for each extraction can be highly problematic. The practice of extracting DNA from some specimens of a population while vouchering others from what appears to be the same population is often used. Unfortunately, without a direct voucher for a definitive identification, it is difficult to determine if unexpected results in a molecular study are correct or if they may be due to contamination, a clerical error, a mixed infestation, or misidentification.

Non-destructive DNA extraction techniques (Favret, 2005; Rowley *et al*., 2007; Rung *et al*., 2008; Dowling *et al*., 2010) have shown promise for preserving the direct voucher specimen without comprising the extraction process. The use of filter paper has been tested as an alternative storage method for insect DNA (Harvey, 2005; Owens & Szalanski, 2005). While this alternative storage method is relatively new for entomology, filter paper has been used in the medical field since the 1970s

(Garrick *et al*., 1973). The current project explores the feasibility of combining a non-destructive DNA extraction technique for voucher preservation, using filter paper as an alternative to deep freeze DNA storage protocols, and augmenting these techniques for specimens stored on microscope slides in specimen collections. Aphids are used for the test insects.

Materials and methods

The specimens used in this study were all apterous adult females from four aphid species collected into 95% ethanol: *Aphis nerii* Boyer de Fonscolombe was collected from *Asclepias tuberosa* L. in Beltsville, Maryland, USA; *Aulacorthum solani* (Kaltenbach) was a USA port of entry interception on *Callistephus chinensis* (L.) Ness from Colombia; *Ovatus crataegarius* Walker was a USA port of entry interception on *Satureja hortensis* L. from Mexico; and *Prociphilus* sp. were collected in Baltimore, Maryland, USA from the roots of *Aster* x *dumosus*. Specimens were stored in 95% ethanol at −80 °C, until the DNA extractions were performed. Subsequent to DNA extractions, the specimens were mounted in Canada balsam (Favret, 2005) and added to the US National Aphid collection housed at the Beltsville Agricultural Research Center, Maryland, USA.

Qiagen Blood & Tissue Kits® (Qiagen, Germany) were used for the DNA extractions. The extractions followed the 'Purification of Total DNA Animal Tissues' protocol with a few modifications (Qiagen®, 2006). First, instead of the whole insect being homogenised, the integument of the aphid was pierced using a minuten insect pin. Secondly, the initial cell lysis step was allowed to continue for approximately 24 h instead of the recommended 1–3 h. This extra time was needed to clear the specimen for microscope slide mounting. Thirdly, for extracts that were to be stored on filter paper, the transfer of the DNA to the filter paper occurred at one of two stages. For some of the samples, the genomic DNA was extracted, purified, and suspended in AE buffer following the manufacturer's protocol. Then the suspended DNA was transferred to filter paper in 35 µl increments and the paper was allowed to dry between applications. Alternatively, the DNA was transferred to filter paper after the initial cell lysis step (after step 2 in the Qiagen® protocols, prior to use of spin columns). These cell lysis solutions were transferred directly to filter paper in 35 µl increments and allowed to dry between applications. In both cases, the extract was added to a one-inch square area of filter paper. The filter paper was kept horizontal and its back was not allowed to touch the substrate until the paper had dried. This was done usually by taping the label end of the filter paper to a box of pipette tips; so that the application area was extended over the edge. The paper was allowed to dry overnight in a fume hood.

Two filter papers were employed: Fisherbrand® Filter Paper Qualitative P5 (Thermo Fisher Scientific, Waltham, MA, USA) (Cat# 09 802 1A) and Whatman® (GE Health, UK) FTA paper. Sheets of Fisherbrand® Filter Paper Qualitative P5 were trimmed to 20 × 29 cm to facilitate application in a HP2200 Deskjet® desktop printer (Hewlett-Packard Corporation, Palo Alto, CA, USA). The locality and collection information recorded on the microscope slide label was also printed in a one square inch area next to the one square inch DNA sample area. An additional one square inch area was left blank for future annotations (Fig. 1). Whatman® FTA paper was also used. However, due to the thickness of the paper, it could not be easily trimmed to size, fed into a desktop printer, or easily attached and stored on the microscope slide.

Trimmed Fisherbrand® filter paper was attached to microscope slides by inserting the paper between two non-reactive polystyrene mounts commonly used by stamp collectors (Hawid® 21 × 24 Clear Mounts, Product number: 024021; Hawid, Germany). Two mounts, oriented in opposite directions and attached to the top surface of the microscope slide, kept the paper firmly secured (Fig. 1).

The DNA that was stored on the filter paper was purified using the Qiagen® 'Purification of Total DNA Animal Tissues' protocol and suspended in AE buffer. Nine 3 mm^2 punches (Fig. 2) were used per purification. The purified product was used to test for the presence of amplifiable DNA.

Specimens of *A. nerii* were initially used to test viability of DNA on filter paper over a duration of time. Testing occurred 1 week, 6 months, 1, and 2 years after the DNA was placed onto the filter paper. Our preliminary studies found that either placing the purified DNA or the solution of the cell lysis buffer with the sample onto the Fisherbrand® P5 or Whatman® FTA paper could be used with similar results (Fig. 3). However, the Fisherbrand® filter paper was selected for further testing because it could easily be trimmed to size for a microscope slide, label data could be printed on it, and the thickness of the paper allowed it to be held in place on the microscope slide by the non-reactive polystyrene stamp mounts.

Further testing used other aphid species and Fisherbrand® filter paper as DNA storage by comparing it with the extraction protocol outlined in the Qiagen® (2006) handbook. Ten specimens each of *A. solani*, *O. crataegarius*, and the *Prociphilus* sp.

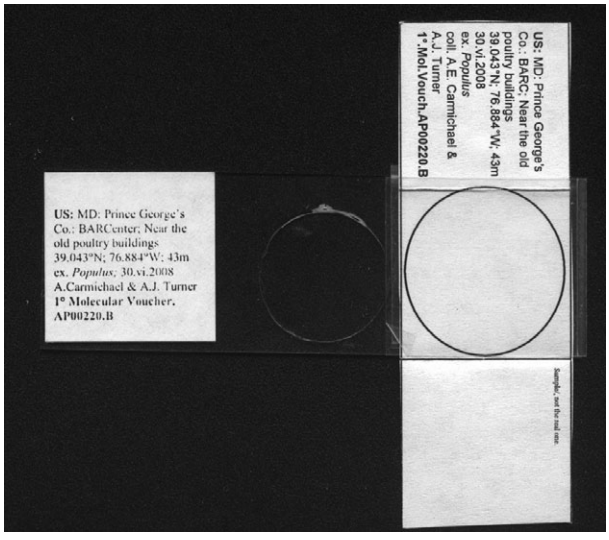

Fig. 1. Voucher specimen (centre) with data label (left side) and DNA filter paper (right side). Polystyrene stamp hinges (right side) are glued to the top side of the microscope slide. Filter paper is shown unfolded for demonstration purposes.

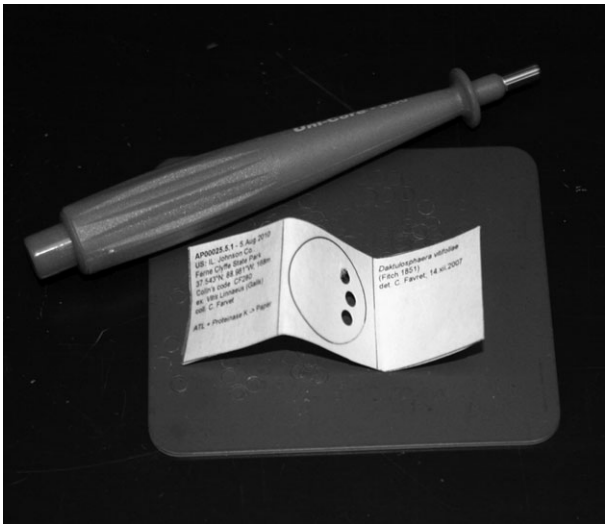

Fig. 2. Example of circumscribed area on the filter paper measuring approximately 20.25 cm^2 (right side) for DNA storage and corresponding collection data or additional information (left side). Cutting mat and 3 mm^2 filter paper punch for removing filter paper.

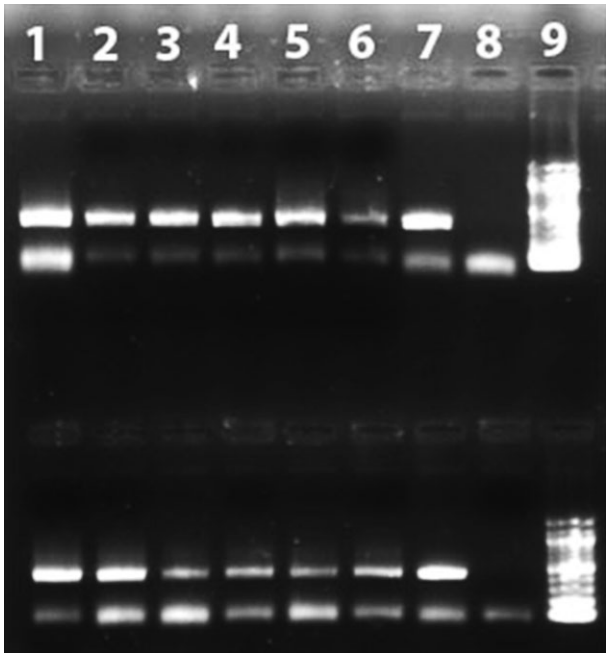

Fig. 3. *Aphis nerii*. A gel of a portion of COII amplified through PCR. The top row represents DNA purification 1 week after the sample was transferred to filter paper, and the bottom row represents purification 1 year after the sample was transferred to filter paper. Lanes 1 and 2 are samples where the cell lysis buffer was transferred to the Fisherbrand® P5 filter paper. Lanes 3 and 4 are samples where the purified DNA was transferred to the Fisherbrand® P5 filter paper. Lane 5 is a sample where the cell lysis buffer was transferred to Whatman® FTA filter paper, and Lane 6 is a sample where the purified DNA was transferred to Whatman® FTA filter paper. Lane 7 is the control sample which was extracted via the Qiagen® DNeasy Kit. Lane 8 is a blank control. Lane 9 is the DNA ladder (BioLabs® 100 bp DNA ladder).

were used. For five of the specimens, half of the extract after the initial cell lysis step was transferred to filter paper, and the remaining amount was purified via the Qiagen® protocol. The other five specimens of each species had their DNA extracted, purified, and suspended in AE buffer. Half of the suspended DNA was transferred to the filter paper. Three months later, 27 cm^2 of the filter paper was used to purify and re-suspend the DNA.

All filter paper samples were stored at room temperature (ca. 20 °C). All DNA samples suspended in AE buffer were stored at −20 °C when not in use.

Presence of DNA was tested for by the amplification and sequencing of a approximately 300 bp region of COII using the forward primer C2-J-3400 (Simon *et al*., 1994) and reverse primer C2-N-3772 (Bogdanowicz *et al*., 1993). Two overlapping regions of COI (each about a approximately 600 bp) were amplified and sequenced using the primer combinations C1-J-1490 and C1-N-2198 (Hajibabaei *et al*., 2006) and COI forward and reverse primers (Favret & Voegtlin, 2004). An example sequence for each gene region and species has been deposited in GenBank (See Table 1).

Attempts were also made to recover EF-1α from the filter paper using same procedure using EF-6 and EF-3 (von Dohlen *et al*., 2002).

Results and discussion

Two segments of mitochondrial DNA were successfully amplified in the initial testing that involved *A. nerii*. The sequence data amplified from the filter paper matched those of the control and sequence data from both control and filter paper matched those for *A. nerii* found in GenBank (HQ528257.1, EF591598.1, DQ499027.1). Results from extractions 1 week and 1 year after initial transfer to the filter paper are shown in Fig. 3. Similar results were also achieved 2 years after initial transfer.

All of the re-extractions of *A. solani*, *O. crataegarius*, and *Prociphilus* sp. worked in amplifying the shorter COII gene region, and the barcoding region of COI was recovered in 5/5 *A. solani*, 5/5 *O. crataegarius*, and 4/5 *Prociphilus* sp. when the cell lysis buffer was transferred to the filter paper. The barcoding region of COI was recovered in 4/5 *A. solani*, 5/5 *O. cratargarius*, and 4/5 *Prociphilus* sp. when the purified DNA was transferred to the filter paper. Results for the above species are from extractions 90 days after initial transfer to the filter paper. See Fig. 4 as example for *A. solani*.

Preliminary results for the recovery of EF-1α from the filter paper storage were partially successful. PCR reactions using the primer pair EF-3 and EF-6 produced similar bands with DNA recovered from the filter paper versus DNA recovered using the Qiagen® protocols. However, the success rate was less with the filter paper (i.e. only 4/15 samples where the cell lysis buffer was placed directly to the paper produced bands, and 8/15 samples where the purified DNA was placed on the paper produced

Table 1. Aphid species and corresponding GenBank accession numbers.

Aphid species	Cytochrome *c* oxidase subunit I (including barcoding region)	Cytochrome *c* oxidase subunit II
Aphis nerii Boyer de Fonscolombe	JF969254	JF969258
Aulacorthum solani (Kaltenbach)	JF969253	JF969257
Ovatus crataegarius Walker	JF969255	JF969259
Prociphilus sp.	JF969256	JF969260

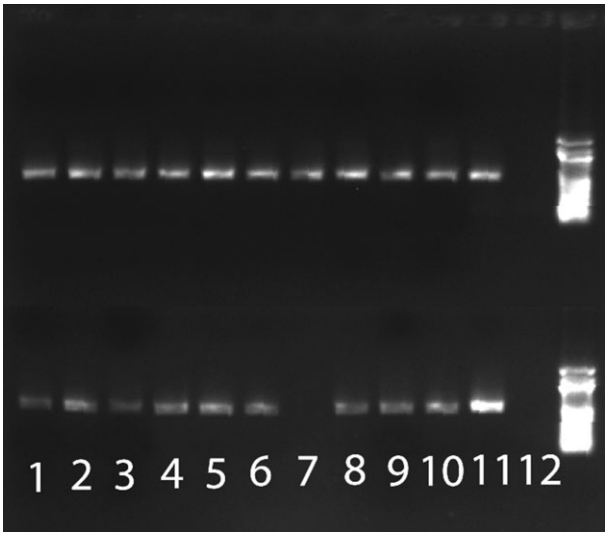

Fig. 4. *Aulacorthum solani*. A gel of the barcoding region of COI amplified through PCR. The top row represents the proportion of the extractions that was extracted normally using Qiagen's protocols. The second row represents extracts from the same individuals as the top row that were transferred to paper for storage for 3 months before being re-extracted. Lanes 1–5: the transfer occurred after the initial cell lysis, and lanes 6–10: the transfer occurred after the DNA had been purified. Lane 11 is a positive control, and Lane 12 is a negative control. Lane 13 is the DNA ladder (BioLabs® 100 bp DNA ladder).

bands). Of the extracts that followed the standard Qiagen® protocol 26⁄30 produced bands. A BLAST search on GenBank confirmed that our sequences were from aphids. These results provide evidence that it is possible to amplify EF-1α from filter paper extractions, although further refinements of EF-1α amplification protocols are needed.

These results support the proof of concept that mitochondrial DNA can be stored with its associated microscope slide-mounted voucher specimen. Furthermore, our study suggests that the voucher specimen's associated DNA need not be purified when initially stored on the filter paper. Rather, the solution of the cell lysis buffer can be applied to the filter paper and purified at a later date if required. The voucher specimen can be examined at any time and stored by conventional means. This methodology could be particularly useful in high output situations (e.g. DNA barcoding endeavours) that generate large quantities of specimens and extracts.

Filter paper storage of DNA has several major advantages over the traditional method of ultralow temperature: (i) The filter paper system allows for DNA storage directly with the curated specimen, thus facilitating its accessibility. As the voucher specimen is directly associated with the DNA and the filter paper is printed with the corresponding locality and identification data that appear on the slide label, cross-referencing is improved. Locating specimens stored in ultra low freezer can sometimes take time depending on the storage and tracking system; (ii) Size of the ultra low freezer requires floor space that can be a premium in a laboratory situation. Use of the filter paper augments the current system used for microscope slide storage; (iii) Freezers require energy to maintain a set temperature and the number of door openings may impact this directly. Additionally, refrigeration systems emit heat into the laboratory, which can affect ambient temperature. Refrigeration systems require safeguards (e.g. backup generator systems) should the main power system fail. Conversely, the filter paper system does not require any additional energy beyond the requirements for normal building maintenance and no additional 'backup systems' are needed. Furthermore, the cost for the Fisherbrand® P5 filter paper is very inexpensive when purchased in bulk sheets and trimmed for use in a desktop printer (at $0.05 per 70 mm × 24 mm paper); (iv) Shipping specimens or extracted DNA stored in ultra low freezers requires special packaging that includes freeze sleeves or dry ice. Filter paper can be shipped in a regular mailing envelope; (v) Using non-reactive polystyrene stamp mounts and filter paper can be incorporated into current microscope slide storage systems (e.g. trays or slide boxes) without any cost for modification; and (vi) DNA preserved on filter paper provides a means send DNA for study without mailing fragile or valuable specimens. While ultra-low temperature is still considered the ideal condition for DNA storage (Vink *et al.*, 2005), use of filter paper at room temperature shows promise and has many distinct advantages. The filter paper system could also be modified in other specimen storage systems. For example, the filter paper could be attached directly below a pinned insect specimen (i.e. enclosed within a gelatin capsule) as part of the routine collection labelling system. We will continue to re-extract DNA at future intervals and test for viability for long-term storage with microscope slides as well as continue to evaluate and test the viability of nuclear DNA.

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