Preparation of Mealybugs (Hemiptera: Pseudococcidae) for Genetic Characterization and Morphological Examination

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ABSTRACT. Mealybugs (Hemiptera: Pseudococcidae) are economically significant agricultural pests on many different crops. Because of their small size and lack of easily visible characters for identification, determination of their taxonomic status is difficult and requires technical competency to prepare a slide-mounted specimen. The standard mounting technique does not allow for analysis of the genome of the specimen. Conversely, preparatory techniques for genetic analysis of mealybugs cause either loss of the entire individual or physical damage that can make morphology-based identification difficult. This study describes a simple protocol that does not impact physical integrity of the specimen for fixation and microscopic examination yet enables simultaneous DNA extraction for DNA-based identification of four mealybug species. All species prepared yielded high quality slide mounts, identified as Planococcus citri Risso, Pseudococcus viburni Signoret, Rhizoecus kondonis Kuwana, or Rhizoecus californicus Ferris. DNA extracted in this manner had higher purity and yield in the final eluate than in samples extracted using standard methods. DNA extracted was successfully amplified by polymerase chain reaction using primers for the cytochrome oxidase I gene and subsequently sequenced for all specimens. This protocol is likely to be applicable to other Hemiptera taxa that are preserved by slide mounting, allowing for both the preparation of a high-quality voucher specimen for morphological identification and simultaneous analysis of DNA for the same specimen. The methods used are technically less challenging than current standard procedures.

Key Words: mealybug, taxonomy, identification, slide-mount, DNA extraction

Mealybugs (Hemiptera: Pseudococcidae) are the second largest family of scale insects, with approximately 2,000 described species in more than 270 genera (Ben-Dov et al. 2003). The family Pseudococcidae has a worldwide distribution but is more common in the subtropics and tropics (Ben-Dov 1994). Their name derives from a white, waxy secretion found on the bodies of adult females and nymphs of most species. Many members of this family are pests of a wide variety of crops grown in tropical, subtropical, and temperate regions, with some species causing significant impact on yield and quality. Economic crop losses occur from large populations of mealybugs and excessive production providing instructions for slide-mounting scales and mealybugs (http://www.ars.usda.gov/SP2UserFiles/Place/12754100/IDService/scale-slides.pdf), with variations of this standard protocol mentioned in many additional publications (Gullan 2000, Downie and Gullan 2004, Triplehorn and Johnson 2005, Malausa et al. 2011). Although this technique yields high-quality slide mounts, it damages the specimen, requires manipulation to clear body contents that takes a high level of skill, and results in the loss of genetic material. Herein we describe a novel and easy-to-use technique to extract DNA from adult female mealybugs while maintaining a fully intact specimen that can be slide mounted without suffering external morphological damage that is suitable for serving as a physical voucher for the exact sequence data to support research or extension activities requiring mealybug identification to species. The technique developed will hereafter be termed the “EPED protocol” (extended proteinase and extended detergent).

Materials and Methods

Sample Collection. Four mealybug species were selected for this study. Planococcus citri Risso and Pseudococcus viburni Signoret were collected from various plants in greenhouses at the University of California, Davis, CA. Specimens of Rhizoecus kondonis Kuwana and Rhizoecus californicus Ferris were collected from soil samples and were observed feeding on grass roots in a vineyard from Oakville, CA. These species were tentatively identified on-site.

DNA Extraction and Clearing of Specimens. For both P. citri and Ps. viburni, 10 adult females were used for extraction of DNA and

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**Results and Discussion**

DNA yield data for samples of *P. citri, Ps. viburni* extracted using the standard protocol, that required complete maceration of samples and for the EPED protocol are presented in Table 1. DNA purity data for samples of *P. citri, Ps. viburni* extracted using the standard protocol, that required complete maceration of samples and for the EPED protocol are presented in Table 2. There was a statistically significant difference between the two extraction protocols for both DNA yield between *P. citri* and *Ps. viburni* and DNA purity in the final extract for both *P. citri* and *Ps. viburni*. On average, extractions conducted using the EPED protocol yielded 10.6 ng/µl more DNA for *P. citri* and 16.8 ng/µl more DNA for *Ps. viburni* than the Qiagen protocol and more pure eluate for both species. Although the difference in actual DNA yield was higher in the EPED protocol, the values obtained from the Qiagen protocol are high enough for amplification by PCR and suitable for research purposes. The higher purity of DNA in the final extract produced by this protocol than that of the standard protocol is highly significant and appears to produce higher quality PCR results (Fig. 1). The 260/280 ratio measurement is an evaluation of the contaminants present in the eluate with accepted ratios to be between 1.8 and 2.0 for DNA (T009-Technical Bulletin, Thermo Scientific, Waltham, MA). The high values presented in Table 1 for both species are likely due to residual cellular components that were not completely cleaned out and, possibly, from minute fragments of exoskeleton present from maceration that do not exist in the eluate produced from the EPED protocol. Regardless of the nature of the contaminants, they likely contributed to lower quality PCR that included weaker signals and nonspecific bands in many of the samples (Fig. 1), whereas samples amplified from eluate produced from the EPED protocol produced relatively strong symbols and produced only one band of the expected size (Fig. 1). Although the extraction methods could not be compared for the two species of *Rhizoecus*.
examined in this study, sufficient DNA of adequate purity was produced by the EPED protocol to allow for successful amplification for these specimens (Fig. 2). All specimens of *R. californicus* and *R. kondonis* yielded substantially lower quantities of DNA per μl of eluate than did extractions of *P. citri* and *Ps. viburni* but much stronger signals were produced in the PCR reactions when compared with the positive controls of *P. citri* and *Ps. viburni* that were extracted with the Qiagen protocol (Fig. 2). This is also an indicator that a high degree of DNA purity in the final eluate is essential for efficient and consistent PCR reactions. Complete clearing of an individual after lysis and dissolving of wax and fat bodies is shown in Fig. 3.

For all specimens prepared with the EPED protocol, a series of slide-mounted vouchers was obtained for *P. citri* (Fig. 4), *Ps. viburni* (Fig. 5), *R. californicus* (Fig. 6), and *R. kondonis* (Fig. 7) with all relevant morphological characters necessary for identification by visible keys and terminology provided by McKenzie (1967) (Fig. 8). The two species collected from greenhouses were identified as *P. citri* and *Ps. viburni*, and the specimens collected from soil samples were identified as *R. californicus* and *R. kondonis*. Morphological identification made on these voucher specimens matched identifications based on comparison of COI sequence data for two of the four species examined in this study. Sequence data obtained from vouchers identified as *P. citri* (KR014243) shared 99% identity with *P. citri* sequences available on GenBank (JF714160.1). Sequence data obtained from vouchers identified as *Ps. viburni* (KR014244) shared 100% identity with *Ps. viburni* sequences available on GenBank (JF714166.1). The two species where

| Species        | Qiagen protocol | EPED protocol |
|----------------|-----------------|---------------|
|                | *n* | Range       | Mean ± SE | *n* | Range       | Mean ± SE | F test  | *P*       |
| *P. citri*     | 10  | 3.99 to 36.36 | 7.70 ± 3.24| 10  | 1.73 to 2.05 | 1.90 ± 0.03| 32.078  | <0.0001  |
| *Ps. viburni*  | 10  | 0.99 to 11.36 | 3.87 ± 1.04| 10  | 1.84 to 2.11 | 1.98 ± 0.03| 32.912  | <0.0001  |
| *R. californicus* | 0   | NA           | NA        | 4   | 1.54 to 2.20 | 1.87 ± 0.14| NA       | NA       |
| *R. kondonis*  | 0   | NA           | NA        | 2   | 1.51 to 1.74 | 1.63 ± 0.12| NA       | NA       |

Extractions used the whole body of adult females.

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**Fig. 1.** Gel electrophoresis of amplicons produced from DNA extracted from mealybugs using standard DNA extraction protocol and the EPED protocol; samples present under EPED correspond to voucher specimens in Figs. 4 and 5, first (+) = plasmid with COI region of *P. citri*, second (-) = plasmid with COI region of *Ps. viburni*, (-) = water control, M = 1 kb + ladder.

**Fig. 2.** Gel electrophoresis of amplicons produced from DNA extracted from *R. californicus* (Rca) and *R. kondonis* (Rko) using protocol described herein. Pci, amplicon from *P. citri* using Qiagen protocol; Pvi, amplicon from *Ps. viburni* using Qiagen protocol; (+), *Ps. viburni* COI amplicon in plasmid; (-), water control; M, 1 kb + ladder.
sequence data could not be compared were *R. californicus* and *R. kondonis* because sequence data for the COI gene for these species were not present in GenBank, but they were found to share 88% identity with *Ps. viburni* (KJ530622.1) for the same region examined. Sequence data for *R. kondonis* (KR014242) in this study and *R. californicus* for this region of COI represent new barcodes.

Specimens prepared using the EPED protocol yielded similar or higher quality vouchers than specimens prepared using the methods described by the USDA Systematic Entomology Laboratory (Fig. 9). This EPED protocol yields a voucher specimen in about a week that is available for photography and identification and required substantially less time for smaller specimens. Indeed, the actual handling time for a specimen is only about 30 min using the EPED protocol. Once the initial lysis is complete, DNA extraction and PCR can be completed within a day and depending on access to sequencing facilities DNA data can be obtained within a few days of beginning the procedure, allowing for molecular identification to occur before the production of a voucher specimen assuming sequence data is available for the species in question. Table 3 presents a timeline for completing the EPED protocol and the actual time spent handling a specimen during that period. Experienced specialists can likely produce a slide-mounted specimen of equal quality to those presented here as well as provide a visual identification in less time than is necessary with the EPED protocol. Such professionals are not present at most research stations where

**Fig. 3.** Exoskeleton of *Ps. viburni* specimen after being properly cleared (A) and with remnant wax and fat bodies (B).

**Fig. 4.** Image plate for corresponding voucher specimens created for *P. citri* from Fig. 2.

| Species     | Lysis (h) | Clearing (h) | Mounting (min) | Handling (min) |
|-------------|-----------|--------------|----------------|---------------|
| *P. citri*  | 36.4 ± 0.9h | 72.1 ± 0.2h   | 2.63 ± 0.12min | 30.3 ± 0.63min |
| *Ps. viburni* | 70.3 ± 0.7h | 95.4 ± 0.1h   | 1.21 ± 0.05min | 26.2 ± 0.21min |
| *R. kondonis* | 8.5 ± 0.5h  | 2.25 ± 0.25h  | 2.90 ± 0.23min | 35.1 ± 0.41min |
| *R. californicus* | 9.2 ± 0.5h  | 2.40 ± 0.11h  | 2.88 ± 0.15min | 32.5 ± 0.22min |

Handling time represents physical time researcher spent handling specimens during a 7-d period. *P. citri* and *Ps. viburni* time tables represent mean of 10 samples each, presented in Figs. 4 and 5, respectively.
identification is needed for research or extension purposes. The technical skills needed to produce high-quality specimens using the standard protocol takes a substantial amount of time to reach perfection, whereas the EPED protocol requires little technical skill and yields consistently high-quality specimens with accompanying sequence data. While a trained professional could produce a specimen and identify it in less time than that presented in the EPED protocol, when considering shipment of specimens to a specialist for preparation and identification and associated communication of results, using the EPED protocol would likely be suitable for research purposes requiring molecular research, with the additional benefit of maintaining a voucher specimen and rapid dissemination of information.

While the EPED protocol in this article was developed using Pseudococcidae, this technique could easily be modified to accommodate other soft-bodied Hemipteran taxa, such as aphids, whiteflies, phylloxera, and scale insects. Other studies have used whole-body DNA extraction to study armored scales (Morse and Normark 2006) and aphids (Sunnucks and Hales 1996); however, individuals in these
studies were crushed to obtain genetic material and it is unclear if crushed individuals were then mounted for identification or if separate specimens were prepared for slide-mounts while some were crushed for genetic analysis. Malausa et al. (2011) described a technique where the specimen was placed intact into a tube with the lysis time extended to between 5 and 8 h before being cleared using standard practices to prepare a slide-mounted specimen. While this technique also allows for the production of a voucher specimen with its corresponding sequence data, the level of physical manipulation and delicate handling of the specimen necessitates a high degree of technical skill.

The EPED protocol makes it possible for entomologists with little experience in mealybug taxonomy or technical skills to prepare a slide-mounted specimen to obtain an exact match between a genome and a physical voucher specimen thereby saving time and money needed to obtain species identification from a specialist. We hope that this technique can facilitate research that will increase understanding of this important and fascinating taxon.

Fig. 7. Image plate for corresponding voucher specimens created for *R. kondonis* from Fig. 3.

Fig. 8. Common morphological characters used in mealybug identification: triocular pores (A) antennal morphology (B) ocular pores and setae, *Ps. viburni* (C) ocular pores, triocular pores, and oral-collar tubular ducts, *P. citri* (D).
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Fig. 9. Specimens of Ps. viburni prepared using standard USDA Systematic Entomology Laboratory protocol (A) and protocol described in this article (B).