Acarologia 50(4): 479–485 (2010)
DOI: 10.1051/acarologia/20101983

SCANNING ELECTRON MICROSCOPY VOUCHERS AND GENOMIC DATA FROM AN INDIVIDUAL SPECIMEN: MAXIMIZING THE UTILITY OF DELICATE AND RARE SPECIMENS

Ashley P. G. DOWLING[1], Gary R. BAUCHAN[2], Ron OCHOA[3] and Jenny J. BEARD[4]

(Received 17 August 2010; accepted 12 October 2010; published online 22 December 2010)

[1] *Assistant Professor, Department of Entomology, University of Arkansas, 319 Agriculture Bldg, Fayetteville, Arkansas, 72701, USA. adowling@uark.edu*
[2] *Research Geneticist, USDA-ARS, Electron & Confocal Microscopy Unit, Beltsville, Maryland, 20705, USA. gary.bauchan@ars.usda.gov*
[3] *Ron Ochoa, Research Entomologist, USDA-ARS Systematic Entomology, 10300 Baltimore Avenue, Bldg 005 BARC-West, Beltsville, Maryland, 20705, USA. ron.ochoa@ars.usda.gov*
[4] *Postdoctoral Researcher, Department of Entomology, 4112A Plant Sciences Building, University of Maryland, College Park, Maryland, 20742, USA; and Queensland Museum, P.O. Box 3300, South Brisbane, Queensland, 4101, Australia. jjbeard@umd.edu*

ABSTRACT — Specimen vouchering is a critical aspect of systematics, especially in genetic studies where the identity of a DNA sample needs to be assured. It can be difficult to obtain a high quality voucher after DNA extraction when dealing with tiny and delicate invertebrates that often do not survive the extraction procedure intact. Likewise, once a whole specimen has been extracted from, it is no longer useful for scanning electron microscopic examination. This paper discusses the use of a single specimen for both low temperature scanning electron microscopy and DNA extraction. This process allows full documentation of all external characteristics of an organism and ample whole genomic DNA extraction for DNA sequencing.

KEYWORDS — low temperature scanning electron microscopy; LTSEM; vouchering; DNA; mites; arthropods

INTRODUCTION

One of the most critical aspects of systematic studies and also the topic of many publications is proper vouchering of specimens (Funk *et al.*, 2005; Huber, 1998; Knutson, 1984). As systematics has shifted towards a focus on genomic data, the importance for vouchering DNA extracted specimens has increased exponentially. This has initiated a series of studies examining non-destructive methods of DNA extraction in arthropods and other invertebrates (Gilbert *et al.*, 2007; Hunter *et al.*, 2008; Phillips and Simon, 1995; Rowley *et al.*, 2007). It is clear from these studies there is no excuse for lack of useful vouchers when extracting DNA from insects and other hard-bodied organisms.

However, different challenges are commonly encountered when working with small (ranging from 100-400 μm in length), soft-bodied organisms like many mites. Oftentimes, based upon personal observations even gentle agitation and soaking of soft-bodied specimens in a digestion buffer leads to loss of some or all setae, effectively making the voucher unidentifiable beyond genus. Additionally, placing a 150 μm mite into a tube of buffer and then

retrieving it unharmed is not an easy task. These mites are essentially invisible to the naked eye, especially when peering through a plastic tube. Successful recovery of the mite requires observation under a microscope and presents numerous opportunities for mechanical destruction of the specimen. Lastly, many mites possess waxy diagnostic structures over their bodies that are destroyed during digestion.

Mite taxonomy relies on observing mite specimens and associated morphological characteristics under high magnification. Traditionally, mite systematists slide-mount specimens for study under light, DIC, and phase microscopy although, in the early 1970's, acarologists began taking advantage of the magnification and depth of field available through scanning electron microscopy (SEM). Many of these studies demonstrated the utility of SEM for observation of new characters and highlighted the distortion caused by flattening mites on slides (Baker, 1995; Oldfield *et al.*, 1972; Otto, 1999; Schliesske, 1988; Thomsen, 1976). Unfortunately, as with slide-mounting, preparation of specimens for traditional SEM incorporates numerous artifacts associated with desiccation and coating, especially in very soft bodied organisms (Beckett and Read, 1986).

Low temperature scanning electron microscopy (LTSEM) avoids problems associated with desiccation by freezing live organisms in liquid nitrogen and maintaining ultracold temperatures within the SEM unit. This not only preserves body turgidity, but often retains the position of seta and other structures in life-like positions, and captures behaviors such as feeding and reproduction (Achor *et al.*, 2001; Wergin*et al.*, 2000).

As mite systematists have turned to genetic data, they have typically recovered specimens during or after DNA extraction and mounted the remaining cuticle as a voucher (Dabert *et al.*, 2008; Dowling and Oconnor, 2010; Jeyaprakash and Hoy, 2010; Klimov and Oconnor, 2008; Roy*et al.*, 2009). This procedure often works well, although as mentioned above, this can lead to complete loss of setae and other damage to delicate specimens. Although we have not attempted to extract DNA and recover a high quality voucher from a broad diversity of mites, in our experience, this loss of seta and overall deformation of body shape and size is common in the Bdelloidea and Tetranychoidea we commonly work with. On the other hand, Mesostigmata like Laelapidae, which the authors also work on, typically only lose a few setae and little deformity of the body occurs. This highlights the necessity of a specific methodology for dealing with soft-bodied, delicate specimens. Additionally, while most voucher specimens in the above mentioned studies are identifiable, a few are too damaged or degraded to obtain reliable species identifications. This can be extremely problematic when closely related species are found in the same collection and there is no way to be sure which one ended up in the extraction vial without a voucher. However, the real conundrum comes when an acarologist has only one specimen of a very rare species. Does one make a slide to confirm identification and take morphological measurements, thus sacrificing the DNA within the mite? Or does one extract DNA, knowing this mite may not be recollected in the near future and take the chance that a high quality voucher specimen remains, allowing for species identification and measurements? Or lastly, does one cut off a couple of legs, hope characters on the other legs are not damaged, and hope that successful extraction ensues from the legs (a process easier said than done with extremely small mites)? The present paper suggests a simple, yet currently unpublished solution to this microarthropod DNA/voucher specimen dilemma.

MATERIALS AND METHODS

Live specimens of three species were processed for LTSEM examination at the USDA Electron & Confocal Microscopy Unit in Beltsville, MD. These included new species of *Erythraeus* (Parasitengona: Erythraeidae) and *Trichosmaris* (Parasitengona: Smarididae) and specimens of *Raoiella indica* (Prostigmata: Tenuipalpidae). Specimens were assigned a unique identifying number and were transferred to flat specimen holders on top of a supercooled (-196°C) brass bar whose lower half was submerged in liquid nitrogen in a Styrofoam box.

FIGURE 1: Example LTSEM images for vouchering specimens. a) *Erythraeus* sp. whole body (40X); b) *Trichosmaris* sp. posterior dorsal trichobothria (900X); *Raoiella indica*. tarsus (3000X); *Trichosmaris* sp. ventral seta (6000X).

Mites were able to walk a few steps before being frozen solid in a life-like position, at which point the holders were transferred to a liquid nitrogen dewar or directly into the LTSEM unit for sputter coating with platinum and imaging. Since no adhesive is used to secure the mite to the holder, after imaging the dorsal surfaces, the mite can be quickly thawed, removed from the holder, flipped onto its back, refrozen and imaged ventrally. Wergin et al. (2000) describes in detail the steps and temperatures involved during LTSEM preparation and imaging. The following modifications to Wergin et al. (2000) were made: a S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Pleasanton, CA) equipped with a Polaron Polar Prep 2000 (Energy Bean Sciences, East Grandby, CT) cryotransfer system was used. Specimens were thoroughly imaged to include all characteristics of mite external morphology (for examples, see Fig. 1) allowing for species identification and discovery of potentially new character systems. After imaging, sputter coated mites were transferred from specimen holders to 100% EtOH.

Whole genomic DNA was extracted from post-LTSEM mites using Qiagen DNeasy Blood and Tissue Kit following protocols therein with an overnight incubation period. Resulting whole genomic DNA for each specimen was given the same unique number assigned to the specimen before LTSEM imaging, catalogued in the lab database, and placed in the -80°C freezer for long-term storage. Remaining cuticles of the three specimens were removed from the vials post DNA extraction and soaked in KOH for 1 min at 40°C. Mite cuticles were removed from the KOH and mounted in Hoyer's medium.

DNA quantification after extraction was performed using a NanoDrop^{TM} 2000 (Thermo Scientific) with a storage buffer blank. Quantifications were done for the post LTSEM extractions as well as an extraction from an ethanol preserved specimen of *Raoiella indica*.

Cytochrome oxidase I (COI) was amplified in 25µL volume reactions for each LTSEM specimen as well as a negative control using LCOI and HCOI primers (Folmer *et al.*, 1994). PCR reactions were prepared and run according to Dowling et al. (2008).

Three microliters of each PCR product was visualized on a 1.5% agarose test gel. All positive samples were prepared for gel extraction and subsequent sequencing according to Dowling and OConnor (2010).

Sequencing of amplified samples was performed by MacrogenUSA (http://www.macrogenusa.com/). Resulting sequences were analyzed using DNASTAR© Lasergene Seqman software, and forward and reverse sequences were reconciled. BLAST searches against the NCBI databases were made to ensure sequences were in fact from the appropriate mite groups. Sequences were submitted to GenBank under the following accession numbers: HQ423154 (*Erythraeus sp.*), HQ423155 (*Trichosmaris sp.*), EU682442 (*Raoiella indica*).

Results and Discussion

All three specimens positively amplified, displaying bright clean bands on the electrophoresis gel of approximately 400bp in length (Fig. 2).

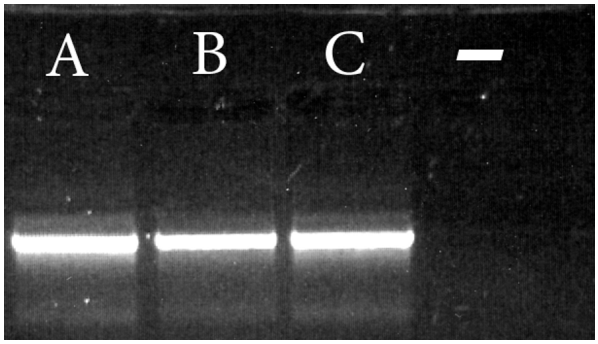

FIGURE 2: Image of agarose gel showing bright bands representing positive amplification of COI. A – *Erythraeus* sp; B – *Trichosmaris* sp; C – *Raoiella indica;* - negative control.

Blast searches for the sequences from the two Parasitengona confirmed identity to that group and the sequence from *Raoiella indica* was identical to previously sequenced *R. indica* samples. There appeared to be no negative effects from the platinum coating on the mite cuticle post-LTSEM, nor any negative effect from the imaging process. The fact that live mites were frozen, stored in liquid nitrogen

and maintained at ultracold temperatures throughout the LTSEM imaging process means that internal tissues (even genetic material of endosymbionts harboured by the mites) should be perfectly preserved (Corthals and DeSalle, 2005). For one of the specimens tested, *Raoiella indica*, we had extractions available from ethanol preserved specimens for direct comparison in terms of DNA quantity post-extraction. The ethanol preserved single specimen had a quantity of 2.5ng/µL whereas the post-LTSEM extraction registered at 4.3ng/µL. Although this shows almost twice as much DNA in the post-LTSEM extraction, because only one set of comparisons were made, no statistical significance can be applied to the results.

However, it has been shown that freezing temperatures are more important to DNA preservation than the preservative used to store specimens (Post *et al.*, 1993; Reiss *et al.*, 1995; Vink *et al.*, 2005). Even specimens stored in 100% EtOH show degradation of low copy nuclear DNA within six weeks if not stored at cryogenic temperatures (Corthals and DeSalle, 2005; Vink *et al.*, 2005).

Based upon the results above, we recommend the combination of LTSEM and DNA extraction be used for dealing with very rare specimens. This approach will provide ample DNA for sequencing and thorough coverage of external morphology for identification and character coding, all from a single specimen. We recognize that LTSEM units are not widely available to all acarologists, however when available LTSEM is to be preferred.

If there is damage to the specimen, the thorough LTSEM coverage should show all necessary characteristics for systematic work. Additionally, this combination is ideal when multiple closely related species are collected in the same sample and cannot be confidently identified under a stereoscope. Our approach would guarantee the association of the individual specimen with the DNA extracted from it.

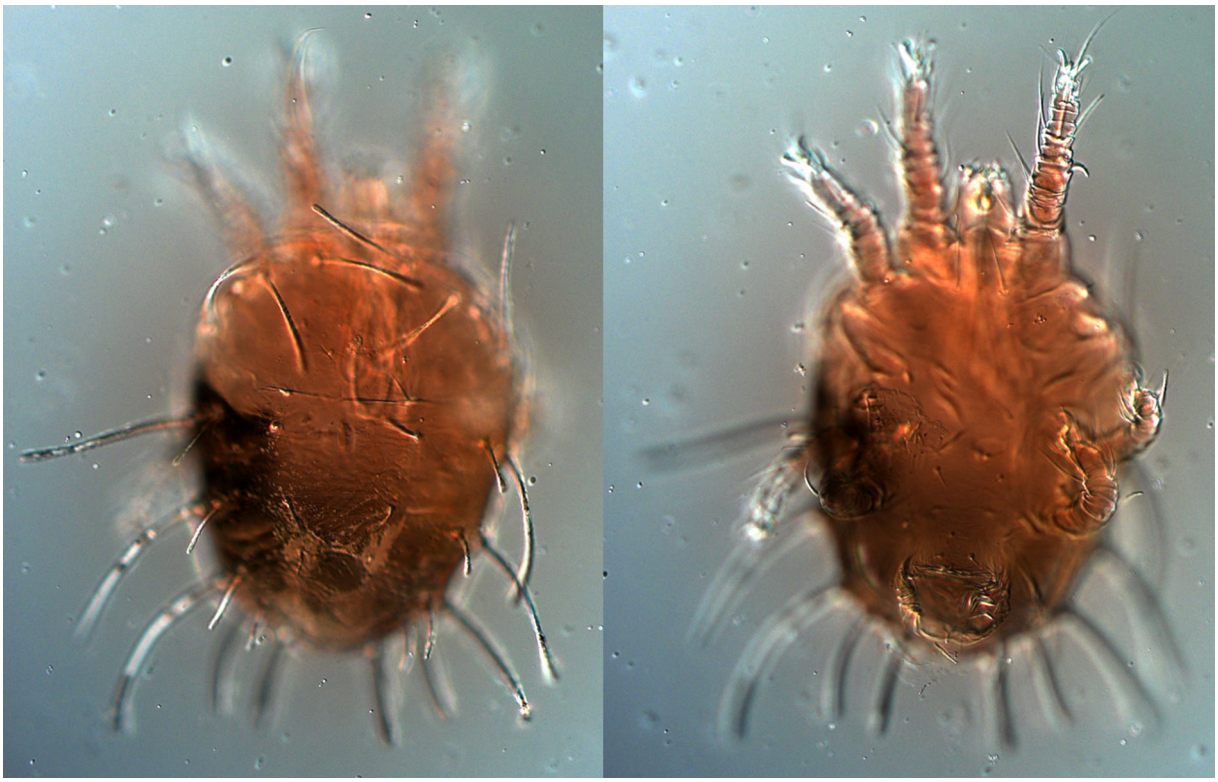

FIGURE 3: Images (40X) of slide mounted *Raoiella indica* specimen (dorsal view on left, ventral view on right) after LTSEM imaging, DNA extraction, and KOH soak.

The remaining cuticle can also be recovered after DNA extraction as we have found that the platinum coating makes the mite a little more durable and more likely to survive the DNA extraction process. Additionally, we have found that soaking the platinum coated mite in KOH after DNA extraction removes most of the platinum coating and produces a decent voucher specimen (Fig. 3). However, we believe the soak in heated KOH removes the platinum coating due to the presence of a waxy layer covering the entire body of the chosen specimens rather than actually affecting the platinum. The thought is that this wax layer runs off of the cuticle, taking the platinum coating with it. We do not believe that all mites possess this waxy layer and therefore the KOH soak may not always remove the platinum coating. These hypotheses need to be confirmed by LTSEM work on other mite groups.

ACKNOWLEDGMENTS

J.J. Beard was partly funded by the Australian Biological Resources Study (ABRS), an initiative of the Australian Government, and by an agreement between USDA-APHIS-PPQ Centre for Plant Health Science and Technology and the University of Maryland. We would like to thank Chris Pooley at the Electron & Confocal Microscopy Unit (ECMU) at the USDA-ARS in Beltsville, Maryland, USA for his work on the plates; Debbie Creel at the Systematic Entomology Laboratory (SEL) USDA-ARS for her continued technical support and reference gathering. We would also like to thank two anonymous reviewers for their helpful comments.

REFERENCES

Achor D.S., Ochoa R., Erbe E.F., Aguilar H., Wergin W.P., Childers C.C. 2001 — Relative advantages of low temperature versus ambient temperature scanning electron microscopy in the study of mite morphology — Int. J. Acarol., 27: 3-12.

Baker A.S. 1995 — A redescription of Halotydeus destructor (Tucker)(Prostigmata: Penthaleidae), with a survey of ontogenetic setal development in the superfamily Eupodoidea — Int. J. Acarol., 21: 261-282.

Beckett A., Read N.D. 1986 — Low temperature scanning electron microscopy — In: Aldrich H.C., Todd W.J., (Eds). Ultrastructure techniques for microorganisms. New York: Plenum Publishing Corporation. p. 45-86.

Corthals A., DeSalle R. 2005 — An application of tissue and DNA banking for genomics and conservation: the Ambrose Monell Cryo-Collection (AMCC) — Syst. Biol., 54: 819-823.

Dabert J., Ehrnsberger R., Dabert M. 2008 — *Glaucalges tytonis* sp. n. (Analgoidea, Xolalgidae) from the barn owl *Tyto alba* (Strigiformes, Tytonidae): compiling morphology with DNA barcode data for taxon descriptions in mites (Acari) — Zootaxa, 1719: 41-52.

Dowling A.P.G., Ochoa R., Beard J.J. 2008 — Preliminary results on phylogeographic patterns of the invasive red palm mite, *Raoiella indica* (Prostigmata: Tenuipalpidae) — In: Bertrand M., Kreiter S., McCoy K.D., Migeon A., Navajas M., Tixier M.S., Vial L., (Eds). Proceedings of the 6th European Congress of Acarology; Montpelier, France. p. 147-154.

Dowling A.P.G., Oconnor B.M. 2010 — Phylogeny of Dermanyssoidea (Acari: Parasitiformes) suggests multiple origins of parasitism — Acarologia, 50: 113-129.

Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R. 1994 — DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates — Mol. Mar. Biol. Biotechnol., 3: 294.

Funk V.A., Hoch P.C., Prather L.A., Wagner W.L. 2005 — The importance of vouchers — Taxon, 54: 127-129.

Gilbert M.T.P., Moore W., Melchior L., Worobey M. 2007 — DNA extraction from dry museum beetles without conferring external morphological damage — PLoS One, 2.

Huber J.T. 1998 — The importance of voucher specimens, with practical guidelines for preserving specimens of the major invertebrate phyla for identification — J. Nat. Hist., 32: 367-385.

Hunter S.J., Goodall T., Walsh K.A., Owen R., Day J.C. 2008 — Nondestructive DNA extraction from blackflies (Diptera: Simuliidae): retaining voucher specimens for DNA barcoding projects — Mol. Ecol. Res., 8: 56-61.

Jeyaprakash A., Hoy A. 2010 — A DNA extraction procedure that allows mite specimens to be slide mounted: phytoseiid species evaluated as a model — Exp. Appl. Acarol., 52: 131-140.

Klimov P.B., Oconnor B.M. 2008 — Origin and higher-level relationships of psoroptidian mites (Acari: Astigmata: Psoroptidia): Evidence from three nuclear genes — Mol. Phyl. Evol., 47: 1135-1156.

Knutson L. 1984 — Voucher material in entomology: a status report — Bull. Entomol. Soc. Am., 30: 8-11.

Oldfield G.N., Newell I.M., Reed D.K. 1972 — Insemination of protogynes of Aculus cornutus from sper-

matophores and description of the sperm cell — Ann. Entomol. Soc. Am., 65: 1080-1084.

Otto J.C. 1999 — Revision of the genus Erythracarus Berlese (Acarina: Anystidae: Erythracarinae) — J. Na. Hist., 33: 825-909.

Phillips A.J., Simon C. 1995 — Simple, efficient, and non-destructive DNA extraction protocol for arthropods — Ann. Entomol. Soc. Am., 88: 281-283.

Post R.J., Flook P.K., Millest A.L. 1993 — Methods for the preservation of insects for DNA studies — Biochem. Syst. Ecol., 21: 85-92.

Reiss R.A., Schwert D.P., Ashworth A.C. 1995 — Field preservation of Coleoptera for molecular genetic analyses — Environ. Entomol., 24: 716-719.

Rowley D.L., Coddington J.A., Gates M.W., Norrbom A.L., Ochoa R.A., Vandenberg N.J., Greenstone M.H. 2007 — Vouchering DNA-barcoded specimens: test of a nondestructive extraction protocol for terrestrial arthropods — Mol. Ecol. Notes, 7: 915-924.

Roy L., Dowling A.P.G., Chauve C.M., Lesna I., Sabelis M.W., Buronfosse T. 2009 — Molecular phylogenetic assessment of host range in Dermanyssus species — Exp. Appl. Acarol., 10: 008-9231.

Schliesske J. 1988 — On the gall mite fauna (Acari: Eriophyoidae) of Cocos nucifera L. in Costa Rica — Plant. Res. Dev., 31: 74-81.

Thomsen J. 1976 — Morphology and biology of the gall mite Eriophyes tiliae tiliae Pgst. (Acarina, Trombidiformes, Eriophyidae) — Ent. Meddr., 44: 9-17.

Vink C.J., Thomas S.M., Paquin P., Hayashi C.Y., Hedin M. 2005 — The effects of preservatives and temperatures on arachnid DNA — Invert. Syst., 19: 99-104.

Wergin W.P., Ochoa R., Erbe E.F., Craemer C., Raina A.K. 2000 — Use of low-temperature field emission scanning electron microscopy to examine mites — Scanning, 22: 145-155.

COPYRIGHT

(cc) BY-NC-ND Dowling *et al.*. Acarologia is under free license. This open-access article is distributed under the terms of the Creative Commons-BY-NC-ND which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original author and source are credited.