Anais da Academia Brasileira de Ciências
ISSN: 0001-3765
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Academia Brasileira de Ciências
Brasil

B. MARTINELLI, ARTURO; WAICHERT, CECILIA; BARBOSA, DIEGO N.; FAGUNDES, VALÉRIA; AZEVEDO, CELSO O.
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Anais da Academia Brasileira de Ciências, vol. 89, núm. 3, julio-septiembre, 2017, pp. 1629-1633
Academia Brasileira de Ciências
Rio de Janeiro, Brasil

Available in: http://www.redalyc.org/articulo.oa?id=32752675021

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Anais da Academia Brasileira de Ciências (2017) 89(3): 1629-1633
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201720160825
www.scielo.br/aabc | www.fb.com/aabcjournal

The use of Proteinase K to access genitalia morphology, vouchering and DNA extraction in minute wasps

ARTURO B. MARTINELLI, CECILIA WAICHERT, DIEGO N. BARBOSA, VALÉRIA FAGUNDES and CELSO O. AZEVEDO

Universidade Federal do Espírito Santo, Departamento de Ciências Biológicas,
Av. Fernando Ferrari, 514, 29075-910 Vitória, ES, Brazil

Manuscript received on November 29, 2016; accepted for publication on April 25, 2017

ABSTRACT

Genitalia are rich source of characters in insect taxonomy. Usually, they are examined after dissection and cleaning with potassium hydroxide (KOH), procedure that may damage both genital morphological structures and intracellular molecular contents. Enzymatic procedure with Proteinase K has been used to clean muscle off the genitalia while extract DNA, but its damage to the genital structures has not been evaluated. Herein, we qualitatively compare the use of KOH and Proteinase K to prepare genital structures in minute insects (Hymenoptera: Bethylidae). We show that Proteinase K is better to preserve the genital structure and provides quality DNA.

Key words: Diaphanization, genitalia preparation, KOH, *Wolbachia*.

INTRODUCTION

Insect genitalia structures are important source of diagnostic characters for several groups of insects. Male genitalia are typically dissected and described after prepared in an aqueous solution of potassium hydroxide (KOH) followed by removal of muscles and fat body (Gurney et al. 1964, Hundsdoerfer and Kitching 2010). The KOH procedure is effective to hasten decomposition of soft tissues and some poorly sclerotized structures. This is true for several groups of Hymenoptera.

Knölke et al. (2005) first proposed extracting DNA as a by-product of genitalia preparation in Lepidoptera, avoiding morphological damage, which is usually achieved when a leg is dissected for DNA extraction. The Proteinase K (PK) treatment preserved morphological traits in Lepidoptera during DNA extraction from genitalia, but the authors did not evaluate efficacy of diaphanization and structure preservation of genitalia after the PK treatment (Knölke et al. 2005, Hundsdoerfer and Kitching 2010). Moreover, Lepidoptera are relatively large specimens and applicability of procedure in minute wasps as parasitoids have not been discussed. Herein, we qualitatively compared the use of KOH and PK protocols to access the genital structure for morphological studies and DNA extraction and amplification using minute parasitoids bethylid.

Correspondence to: Arturo Benincá Martinelli
E-mail: martinelliab@gmail.com

MATERIALS AND METHODS

We used males of *Dissomphalus* Ashmead (~3.4 mm body length, ~0.52 mm genitalia) and *Pristocera* Klug (~5.8 mm, ~1.3 mm) (Hymenoptera: Bethylidae), totalizing 39 specimens. We used only specimens conserved in alcohol and collected in or after 2005 to increase success in DNA extraction and amplification (Knölke et al. 2005). For both KOH and PK digestion protocols, the genitalia were removed from the last metasomal segment with a sterile needle and were stored in polyethylene genitalia vials filled with glycerin.

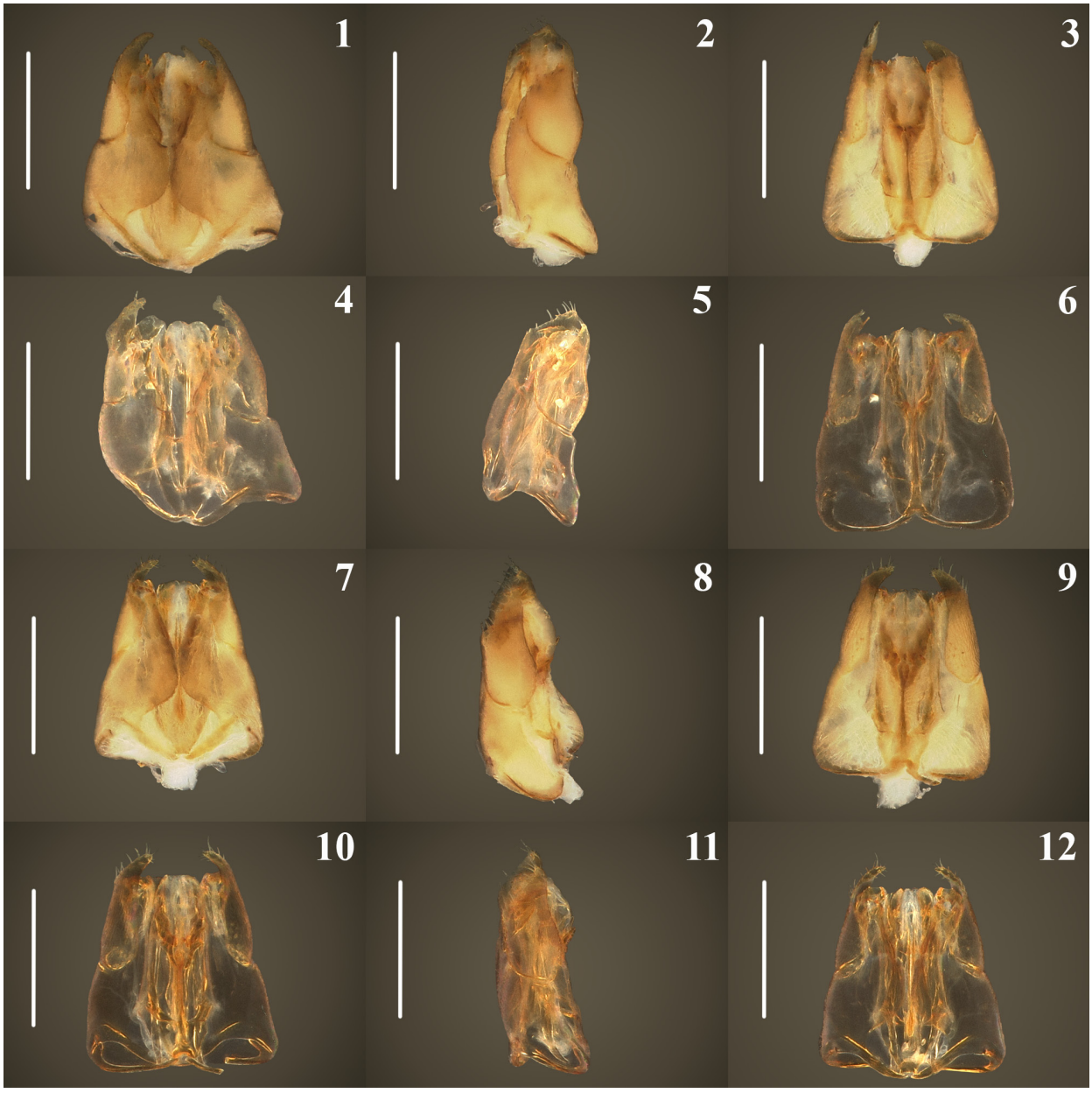
For KOH digestion, the last metasomal segment was submersed in 10% KOH solution in a porcelain spot plate and kept submersed at high temperature until complete digestion of all the soft tissue (5-20 minutes, depending on size, see Results section). High temperature was achieved through Corning Hot Plate Scholar 170. Then, the segment was washed in 25% acetic acid to neutralize the pH and finally, the genitalia, the terga and the sterna were separated by dissection and washed with water to clean remaining tissues.

For PK digestion, the last metasomal segment was placed in a 1.5 mL microcentrifuge tube containing a solution of 5 µL Proteinase K (20 mg/mL) and 25 µL buffer (pH 8.0, 10 mM Tris-Cl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS) using a sterile needle and kept overnight in a hot plate or incubator at 55°C. The genitalia were gently separated from the digestion solution using a pipette, taking care to not pull the genitalia structure into the tip. The solution was transferred to a new tube for further DNA extraction procedures. The tube with the genitalia was kept at 95°C for 10 minutes to inactivate the enzyme and then washed with water. The genitalia resulting from both procedures were photographed in ventral, dorsal and lateral views with the EntoVision® system combined with Helicon Focus®.

In order to test DNA amplification, we extracted DNA from the PK digestion solution using the DNA MACHEREY-NAGEL NucleoSpin® Tissue Kit and following the manufacturer protocol. We amplified the molecular markers mitochondrial *cytochrome C oxidase subunit I* (*COI*) (Folmer et al. 1994), nuclear *internal transcribed spacer 2* (*ITS2*) (Weekers et al. 2001), and *28S ribosomal RNA* (*28S*) (Carr et al. 2010). Polymerase Chain Reaction (PCR) used 50 µL final volume containing 1-4 µL DNA extract, 1x Taq buffer, 1.5 mM MgCl2, 0.8 mM dNTP mix, 0.16 µM of each primer, and 1.0 U Platinum® Taq DNA Polymerase (Invitrogen, Inc.). The initial denaturation was performed at 93ºC for 5 min, followed by 45 cycles of 93°C for 1 min, 46°C (*COI*) or 55ºC (*28S* and *ITS2*) for 45 sec and 72°C for 45 sec, with a final extension at 72°C for 5 min. To evaluate the presence of the amplicon, we ran 3 µL of PCR product on a 1% TBE agarose gel. When agarose gel held almost inconspicuous bands, we performed a second PCR reaction, as described above, replacing the 1-4 µL of DNA extract by 0.5 µL of the first PCR product, as an option to optimize the target DNA amplification. PCR products were purified by an enzymatic procedure with the ExoSAP-IT® kit (USB Corporation) and incubated at 37°C for 15 minutes to degrade remaining primers and nucleotides and at 80°C for 15 minutes to inactivate ExoSAP-IT reagent. Amplicons were sequenced following the Big Dye® Terminator protocol (Applied Biosystems) on an ABI3700 Genetic Analyzer.

RESULTS AND DISCUSSION

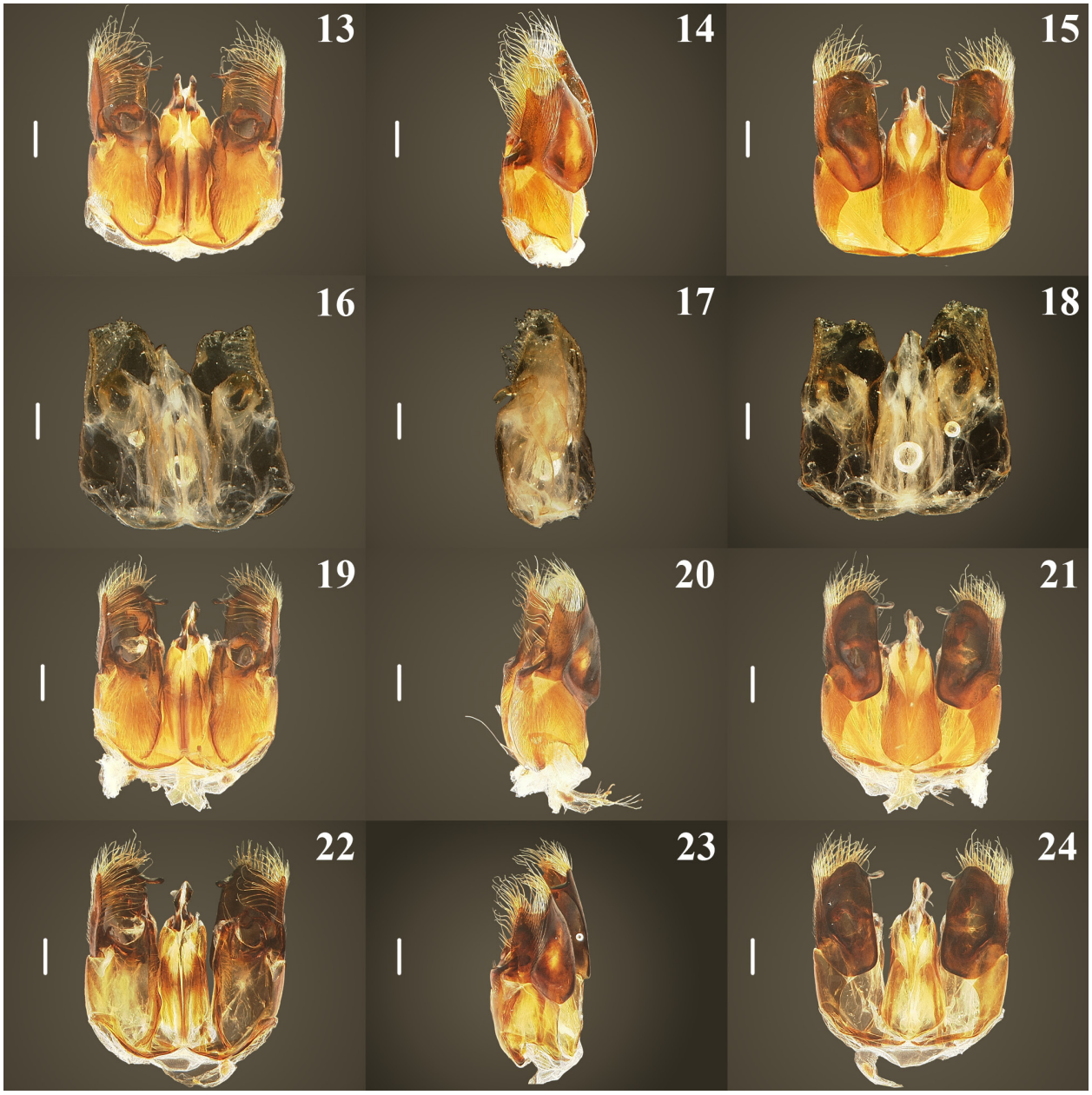
Overall, both protocols (KOH and PK) successfully cleaned the genitalia and allowed access to the most relevant features for taxonomic studies (Figs. 1-24). However, KOH protocol underwent more severe deterioration of sclerotic parts as well as clarified and softened genitalia sclerites. The PK procedure was more efficient in preserve the textured patterns



Figures 1-12- Male genital of *Dissomphalus* sp. Before KOH treatment: **1**. Dorsal view; **2**. Lateral view; **3**. Ventral view. After KOH treatment: **4**. Dorsal view; **5**. Lateral view; **6**. Ventral view. Before Proteinase K treatment: **7**. Dorsal view; **8**. Lateral view; **9**. Ventral view. After Proteinase K treatment: **10**. Dorsal view; **11**. Lateral view; **12**. Ventral view. Scale bars=300 µm.

from the genitalia or other sclerotized structures and provided higher quality images of genitalia structures.

We observed different outcomes in a matter of size of studied specimen. In small individuals (~3.4 mm body length) both protocols had similar results (Figs. 4-6; 10-12); whereas in larger specimens (~5.8 mm) the PK protocol was more suitable to clean and preserve fragile parts of the genitalia, such as sclerites, membranes and setae (Figs. 16-18). The larger and more sclerotized the specimens, the longer the exposure time of KOH treatment (about 10-20 minutes in specimens of *Pristocera*), resulting in degradation of the



Figures 13-24- Male genital of *Pristocera* sp. Before KOH treatment: **13**. Dorsal view; **14**. Lateral view; **15**. Ventral view. After KOH treatment: **16**. Dorsal view; **17**. Lateral view; **18**. Ventral view. Before Proteinase K treatment: **19**. Dorsal view; **20**. Lateral view; **21**. Ventral view. After Proteinase K treatment: **22**. Dorsal view; **23**. Lateral view; **24**. Ventral view. Scale bars=300 µm.

sclerites, shortening of apex of hairs and fading of poorly sclerotized membranes (Figs. 16-18). Inversely, the reduction of KOH exposure time decreased the degradation of sclerite, but favored the maintenance of muscles. Thus, preservation of structures, solution concentration and exposure time might be challenging when using KOH protocol. The loss of specimens by trial and error during standardization represents a constraint if the sample is small or composed by type specimens. In contrast, PK cleaning protocol successfully removed soft tissues and preserved genital features, including poorly sclerotized membranes (Figs. 22-24); maintained the genitalia integument rigid and

preserved, allowing observation of texture; and did not require standardization of exposure time.

We successfully amplified DNA from 37 out of the 39 wasp specimens. The full dataset (three genes) was recovered in seven specimens (18.9%), while 23 bethylids (62.1%) were sequenced for *ITS2* (650-900 bp), 27 (72.9%) for *COI* (630 bp), and 22 (59.4%) for *28S* (750 bp). The amount of DNA obtained from the PK treatment was reduced (<5ng/µL), but was enough to amplify both mitochondrial and nuclear genes. A second round of PCR optimized the amplification, but it increased cross-amplification of the endosymbiont *Wolbachia*, which is herein first recorded for Bethylidae. The detection of *Wolbachia* with universal COI primers is a common issue reported in the literature for other invertebrates (Smith et al. 2012).

Finally, by adopting the PK protocol here described, we were also able to extract and amplify DNA from genitalia previously preserved in microvials filled with glycerin. Glycerin preserves tissues better than dryness (Quicke et al. 1999) and does not inhibit PCR reagents. It could be a source of genomic data even in destroyed collection exemplars.

Concluding, the PK procedure has advantages, which favored it over the widely used KOH protocol: 1) it allows access to genitalia morphology without damaging the diagnostic characters; 2) it is feasible to extract and amplify DNA; 3) the DNA can be used for barcoding; and 4) it avoids loss of individuals to morphological analyses.

ACKNOWLEDGMENTS

We thank Rodrigo B. Ferreira and anonymous reviewer for comments on manuscript and Juliana F. Justino from Núcleo de Genética Aplicada à Conservação da Biodiversidade (NGACB) at Universidade Federal do Espírito Santo (UFES) for logistical support. This work was funded by Fundação de Amparo à Pesquisa e Inovação do Espírito Santo (FAPES) (#52263010/2011 to COA) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (grants #305746/2014-6 to COA and #620068/2008-4 to VF). Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) provided MS scholarship to ABM and DNB and postdoctoral fellowship to CW (Process #71026436). This manuscript was revised by American Journal Experts, with funds from UFES.

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