



## Short Communication

## Clearing and dissecting insects for internal skeletal morphological research with particular reference to bees



Diego Sasso Porto<sup>a,\*</sup>, Gabriel A.R. Melo<sup>b</sup>, Eduardo A.B. Almeida<sup>a</sup>

<sup>a</sup> Laboratório de Biologia Comparada e Abelhas, Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil

<sup>b</sup> Laboratório de Biologia Comparada de Hymenoptera, Departamento de Zoologia, Universidade Federal do Paraná, Curitiba, PR, Brazil

## ARTICLE INFO

## Article history:

Received 20 July 2015

Accepted 4 November 2015

Available online 1 December 2015

Associate Editor: Rodrigo B. Gonçalves

## Keywords:

Apidae

Apoidea

Comparative morphology

Hymenoptera

Internal morphology

## ABSTRACT

A detailed protocol for chemical clearing of bee specimens is presented. Dry specimens as well as those preserved in liquid media can be cleared using this protocol. The procedure consists of a combined use of alkaline solution (KOH or NaOH) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), followed by the boiling of the cleared specimens in 60–70% EtOH. Clearing is particularly useful for internal skeletal morphological research. This procedure allows for efficient study of internal projections of the exoskeleton (e.g., apodemes, furcae, phragmata, tentoria, internal ridges and sulci), but this process makes external features of the integument, as some sutures and sulci, readily available for observation as well. Upon completion of the chemical clearing process the specimens can be stored in glycerin. This procedure was developed and evaluated for the preparation of bees and other Apoidea, but modifications for use with other insect taxa should be straightforward after some experimentation on variations of timing of steps, concentration of solutions, temperatures, and the necessity of a given step. Comments on the long-term storage, morphological examination, and photodocumentation of cleared specimens are also provided.

© 2015 Sociedade Brasileira de Entomologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

The study of the morphology of different kinds of organisms, especially animals, certainly is one of the easiest, cheapest, and most readily accessible ways of obtaining large amounts of information and understanding of the planet's biodiversity. Morphological comparative studies have had a long life, as exemplified by the classic treatise *De Partibus Animalium* by Aristotle more than 2300 years ago. In the case of Entomology, external and internal morphological studies have always had great appeal because of the enormous diversity of insects, but also because of large amount of variation observable in their exoskeleton. Despite the emergence of new techniques for imaging complex insect anatomy such as  $\mu$ CT-Scan, MRI, CLSM (e.g., Beutel and Friedrich, 2008; Deans et al., 2012), traditional light optical microscopes remain as the most common instruments of assessing morphological diversity. Specimen dissection and preparations for morphological research using optical microscopy is benefited by clearing the cuticle using alkaline solutions, such as NaOH or KOH. Clearing is particularly useful when internal projections of the exoskeleton (e.g., apodemes, furcae, phragmata, tentoria, internal ridges and sulci) are investigated, but this process makes external features of the integument, as

some sutures and sulci, more readily available for observation. Furthermore, cleared material is appropriate for line drawings and photography, in addition to remain available for posterior preparation for SEM execution after being critical pointed dried if desired (Porto et al., 2015).

Although alkaline solutions (e.g., NaOH or KOH) have been commonly used in entomological clearing procedures by researchers working with various insect taxa, protocols combining treatments with these kinds of bases and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) are not usually done. Melo (1999) reported the combined application of an alkaline compound (KOH) and a solution intended to make the insect integument more translucent (H<sub>2</sub>O<sub>2</sub>). The clearing procedure presented by Melo (1999) contained the general steps but a protocol comprising detailed approximate times and alternative routes for these steps into a more readily applicable protocol appears to be lacking. A modified version of the Melo's (1999) clearing method is provided in Table 1. Our protocol was developed and evaluated for the preparation of bees (and other Apoidea, as done by Melo (1999)), but modifications for other insect taxa should be straightforward after some experimentation on variations of timing of steps, concentration of solutions, temperatures, and the necessity of a given step. The understanding of the relationships between the main lineages of bees has been greatly improved during the XX century by many morphological works, particularly those by

\* Corresponding author.

E-mail: [diegosporto@gmail.com](mailto:diegosporto@gmail.com) (D. Sasso Porto).

**Table 1**  
Protocol containing the suggested steps for clearing and dissecting specimens of bees (and other relatively large and well sclerotized hymenopteran representatives) for the study of external and internal skeletal morphology (modified from Melo, 1999).

(1) Place the specimen into a 10% solution of KOH/NaOH to dissolve soft tissues. The specimen may remain soaked in solution for 10–45 h, in room temperature. This step of the procedure can be speed up by heating the KOH/NaOH solution up to ~90–100 °C, preferably in a water bath, in which case the time needed for the initial clearing can be reduced to 10–40 min. The necessary time for heated KOH/NaOH to be effective depends on various traits of the specimen, such as body size, color, thickness of the integument, region of the body.

Dry pinned specimens do not need to be relaxed before being placed into the KOH solution, although this might be necessary to safely remove the specimen from the pin. In case the specimen is preserved in ethanol (70–80%), boil it in 100% EtOH for approximately 10 min, then transfer it to heated KOH/NaOH and proceed as indicated in the standard protocol. Overall, no significant differences between ethanol preserved specimens and dry-pinned can be perceived at the end of the clearing process.

Delicate parts of the specimen, such as mouthparts and terminalia, may be dissected/disarticulated at this stage to prevent over clearing them. Additionally, the head and propectus (i.e., propleuron and prosternum), mesosoma and metasoma can be separated prior to the next steps. This allows the fine tune of the clearing process for distinct portions of the body, as they normally have different properties (e.g., cuticle thickness, colors, etc.) and, thus, will probably differ in their ideal times in heated solution.

(2) Transfer the specimen to a room-temperature solution of 3–5% H<sub>2</sub>O<sub>2</sub>, where it shall remain for approximately 10–20 min, when it will be cleared enough. Once again, the necessary time for the clearing to be effective depends on various traits of the specimen, such as size, color, integument thickness, region of the body, etc. Alternatively, a heated (~70–90 °C) solution of H<sub>2</sub>O<sub>2</sub> may be used, but this must be done with caution because the clearing activity might be over accelerated leading to a reduced control over the degree of the overall clearing process. For proper clearing, it is important to emphasize that the specimen needs to be transferred directly from the alkaline solution to the peroxide solution. Do not wash it in water between the two steps.

In certain cases, treatment with KOH/NaOH will be effective enough so that it would be advisable to skip the H<sub>2</sub>O<sub>2</sub> step (in such cases, the chemical action of KOH/NaOH must be stopped, and this can be accomplished with a bath in diluted acetic acid or lactic acid for a few minutes, followed by washing in pure water. Going to step 3 directly from 1 will also work).

(3) Transfer the specimen and all its previously disarticulated parts (if any) to a 60–70% EtOH boiling solution, leaving them there for 5–10 min. There will be bubbles popping out of the body, but this is normal and necessary to remove small air bubbles from within the exoskeleton and remnants of macerated soft tissues as well as to interrupt the chemical effects of KOH/NaOH and H<sub>2</sub>O<sub>2</sub>.

(4) Wait for the solution to cool down to naturally reach room temperature.

(5) Transfer the specimen to a glycerol: 60% EtOH mixture (1:1).

(6) After approximately 24 h, remove part of the supernatant EtOH with a pipet and double the amount of glycerol. Ethanol and glycerol do not mix perfectly well because they possess distinct densities. After some time, it is easily noticeable that there will be more EtOH on the upper layer of this solution.

(7) Wait for the specimen to be completely immersed in the glycerol (this may require a few days), and then transfer the specimen to a pure glycerol solution.

(5–7, alternative) The time required for these steps can be significantly shortened using a series of dehydrating alcoholic solutions. After step 4, the dissected pieces can be soaked into a series of EtOH solutions with increasing concentrations (70–100%) for 20–30 min each. Four baths are sufficient (70%, 80%, 90% and then 100%) for a successful dehydration of the specimens or specimen-parts. After that, store the pieces into pure glycerol.

Roig-Alsina and Michener (1993) and Alexander and Michener (1995), which investigated external and internal skeletal morphology of Apoidea in a comparative manner.

The protocol adopted by Melo (1999) was in current use at the Entomology Division of the University of Kansas Natural History Museum in the 1990s. GARM recalls not learning it from a written document, but mostly from talking to Charles Michener and the late Byron Alexander. He also recalls learning from the late Steve Ashe the step of boiling specimens in diluted ethanol after peroxide clearing to remove the air bubbles from within them. A search in Ashe's publications at that time (e.g., Ashe, 1992; Ahn and Ashe, 1996) reveals that use of hydrogen peroxide to clear dark specimens is indicated, but subsequent boiling in ethanol is not mentioned. A detailed clearing and dissecting protocol for aleocharine staphylinid beetles was later published by Hanley and Ashe (2003) in which this step is mentioned and discussed. Differently from Hanley and Ashe (2003), the protocol detailed in the present paper focuses mostly on preparing specimens that will be kept disassembled permanently in glycerin in individual wells of plastic culture plates, instead of being mounted in permanent slides, as it is done in the work of the former authors.

## Protocol

Prior to starting the chemical clearing process of a specimen, it is advisable to remove its wings. There is no harm in leaving them attached to the specimen, especially when it is expected that a study of its microscopic structure will be carried out, but they will be completely deformed by the procedure described in Table 1. It is recommended that one pair is permanently mounted in glass

slides for study under light microscopy and the other pair is glued in a piece of paper and pinned with the specimen labels, which then can be properly stored for further records.

Useful techniques for preparation of fine dissecting tools are given by Hanley and Ashe (2003). These pin tools are also quite handful when dealing with the whole storage plates (see below) under dissecting microscopes. They allow proper positioning of the insect parts without requiring that the examined part be taken out of the plate wells.

**Step 1 (Table 1)**—Two alternatives are possible at this step: either the specimen can be soaked into a 10% KOH/NaOH solution in room temperature overnight (Melo, 1999: step 1), or this solution can be heated at 90–100 °C. The immersion of specimens in solution by a period of 12–24 h (generally done overnight), although guarantee more control over the clearing/softening process of the integument, showed inefficient results in many instances. On the one hand, for very small specimens (<3 mm), this time proved to be too long and resulted into excessive softening of the integument, making the specimens structurally very weak to manipulations during dissections. On the other hand, for larger specimens (>15 mm), sclerites and articulations are kept structurally intact or only slightly affected, even after more than 24 h in a solution in room temperature.

Heating accelerates the reaction of the alkaline compounds with the integument, membranes, and soft tissues, therefore facilitating the breaking of chemical bonds between the linear chains of chitin and the proteins that are responsible for the rigidity/pigmentation of exocuticle. For specimens with body size between 10 and 15 mm (with moderately sclerotized cuticle) heating the 10% KOH/NaOH solution for about half an hour at 90–100 °C suffices in most

cases—these conditions also create a balance between the structural integrity of the sclerites and the intention of clearing the cuticle. Smaller or more fragile specimens were cleared efficiently in ca. 15–20 min; whereas very large specimens (>25 mm) can generally be safely heated for 45–60 min without evident damage.

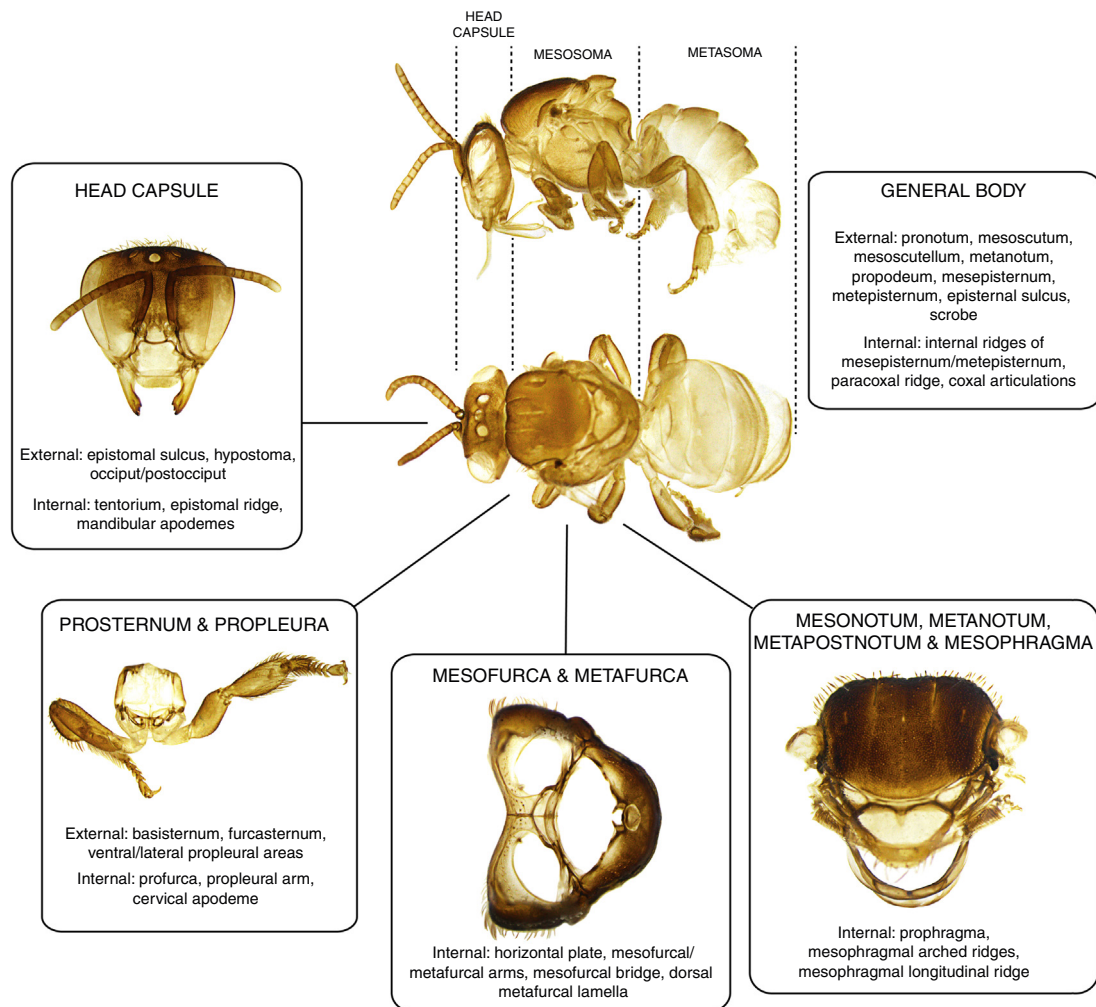
**Step 2** (Table 1)—The second step consists of soaking the dissected pieces of interest into 3–5% hydrogen peroxide ( $H_2O_2$ ). This step is important because it allows additional clearing of the integument without the undesirable excessive softening resulting from the continued action of an alkaline solution. The use of  $H_2O_2$  is indicated for those specimens with dark integument and/or when there is particular interest on the study of internal projections of the exoskeleton. Extending the time the material is soaked into the hydrogen peroxide can improve the clearing of the cuticle making it translucent and allowing the observation of internal hard structures (e.g., tentorium, phragmata, furcae, apodemes, internal ridges and sulci). This process is more efficiently executed with warm hydrogen peroxide. Results can also be observed at lower temperatures, but the clearing will be noticeably slower.

This can be the most challenging step of the protocol and requires careful standardization to prevent excessive clearing, as some specimens or parts of specimens can be made almost transparent after this step. A successful clearing process is generally indicated by a light reddish/brownish coloration of the integument (Fig. 1), although this can vary depending on the specimen. The

integument color (i.e., dark, pale-brown, yellowish, metallic), the overall degree of cuticle sclerotization, and the size of the specimen were the most important factors influencing the results of clearing using  $H_2O_2$ . Distinct regions of the body can be differentially cleared, which oftentimes requires disarticulation of head, mesosoma, metasoma, and of certain other structures (e.g., mouthparts, legs, propleura, prosternum, male genitalia, and sting apparatus).

Mouthparts and genitalia should be separated first from the remainder of the body and cleared individually. Timing for the clearing for these structures varied from 30 to 150 s. The time needed for the  $H_2O_2$  stage was considerably shorter for the head capsule plus the propectus (1–5 min), an intermediate amount of time is optimal for the metasoma (2–7 min), and longest times for the mesosoma (3–10 min). For some structures, as the mesosoma of dark, heavily sclerotized large specimens, the time spent in the warm  $H_2O_2$  solution can be as long as 15–20 min.

**Step 3** (Table 1)—Boiling the specimens in 60–70% EtOH for 5–10 min (depending on the size of the specimen or structure) allows the removal of air bubbles confined into the sclerotized structures or within remnants of partially macerated soft tissues. The second purpose of this step is the neutralization of the actions of KOH/NaOH and  $H_2O_2$ . Specimens boiled at EtOH for less than 3 min tend to have residual clearing resultant from the excessive action of alkaline compounds (KOH or NaOH) after the completion of the entire process.



**Fig. 1.** Specimens of *Scaptotrigona depilis* (Moure, 1942) cleared using the protocol presented in Table 1: lateral habitus (central column, top); dorsal habitus (central column, middle); boxes show different portions of the body disarticulated, along with examples of external/internal structures made available for refined morphological inspections after the clearing procedure.

**Steps 4–7 (Table 1)**—Before the final storage in glycerol, it is important to wait the solution cool down (step 4) to avoid thermal shock, which can damage some fragile structures (e.g., mouthparts, portions of the genitalia, and sting apparatus). The transferring procedure (steps 5–7) to pure glycerol should be made in a stepwise manner, since ethanol and glycerol do not mix perfectly well due to their distinct densities. Some time is required to the 60% EtOH in the mixture goes to the upper phase and can be removed with a pipet. The addition of glycerol can be made once or twice, until almost all the 60% EtOH was removed, before permanent storage into pure glycerol. An alternative to this original procedure outlined by Melo (1999: steps 4–7) is to use a series of dehydrating alcoholic solutions in increasing concentrations (e.g., 70%, 80%, 90%, 100%), soaking the structures about 20 min in each bath, then finally storing then into pure glycerin. This shortens the time required for the storing process in glycerin, instead of using a hydrated ethanol mixture and slow addition of glycerin.

### Specimen storage, manipulation, and photodocumentation

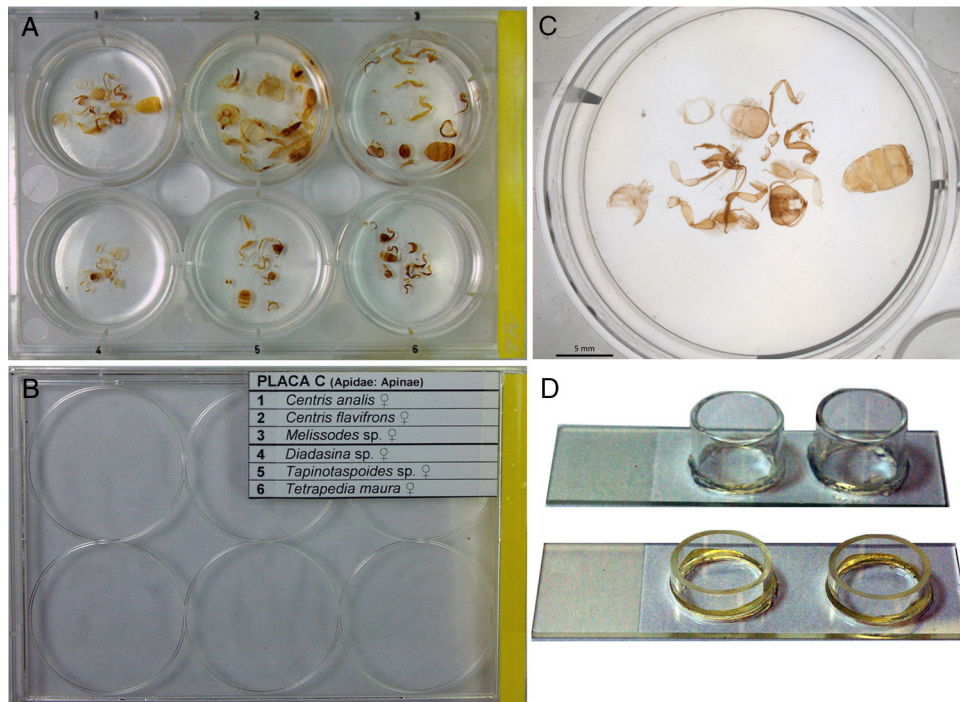
Long-term storage of the cleared and dissected specimens can be easily accomplished by keeping each one in individual wells of acrylic cell culture plates (also termed ‘tissue culture plates’) (Fig. 2A and B). Permanent storage in culture plates imposes certain restrictions for transportation of the specimens. When over short distances, the plates can be easily hand carried as long as they are not strongly tilted or jolted in the process. Under normal conditions, they can also be transported by car if necessary. Long distance transportation, such as in airplanes, should be preceded by sealing of the wells with discs of high-density plastic foam (e.g., polyethylene or polyurethane). Removal of the foam discs should be done carefully to make sure that no dissected parts are taken away adhered to glycerin drops that might have spilled on the discs.

Specimen study under both reflected and transmitted light can also be readily carried out directly on the plates due to their

transparent walls (Fig. 2C). Culture plates are produced with wells of distinct diameters and they should be chosen according to the size of the specimens. However, the minimum diameter of the individual wells should not be less than 15 mm, even when used to store small specimens. Smaller diameters make it difficult to move the dissected specimen parts while simultaneously observing them under a dissecting microscope. In some instances, when the focal distance of the microscope is very short, these plates can make manipulation of the specimens difficult. In this case, the use of manipulative tools – as a wooden stick with a pin attached to its tip or something similar – can help to overcome the problem of a short focal distance. Alternatively, the specimens can be transferred to ceramic or glass well-plates, but the former does not allow the use of transmitted light and the latter is often expensive. In the latter case, a small Petri dish can be a more economical alternative. Examination with concomitant manipulation of several specimens from a single plate or from different plates requires careful attention to avoid that observed parts are not mixed up between wells. If not strictly observed, specimen parts can remain attached to droplets of glycerin at the tips of the manipulation tools.

The translucent property of many liquid media, particularly glycerol, makes cleared specimens and their parts available for optical inspections, line drawings (with aid of a *camera lucida*), or photodocumentation. During morphological investigation and when using a *camera lucida* for line drawings, the contrast can be greatly enhanced by altering the technique of light transmission, varying between bright field and dark field.

Specimens can be photographed immersed in pure glycerol inside the wells of the culture plate, but this procedure is not recommended. Although the optical properties of the glycerol are generally good enough for photography due to its translucent nature and its flat surface without undulations (which makes no reflections to the light), its density is not as high as some other colloidal media, such as water-soluble commercial personal lubricating jelly or a 1:1 mixture of glycerol and gelatin. The chosen



**Fig. 2.** Accessories for specimen storage, study and photodocumentation: (A) acrylic cell culture plate with six round wells used for storing specimens in glycerol or other liquid media after the clearing procedure; (B) plate-cover with an example of a labeling system for specimens stored in the six wells; (C) close-up view of one well (diameter = 35 mm) containing a cleared and disarticulated female specimen of *Centris analis* Lepeletier, 1841; (D) modified glass slide used for accommodating specimens, which can be filled with high density colloidal medium during photodocumentation.

medium can be put into an excavated glass slide for microscopy with the specimen or structure of interest properly positioned. A small droplet of glycerol shall be added onto the top of the water-soluble lubricant to smoothen the undulated surface and reduce the light reflection on its surface. A cover slip should then be placed above the immersed specimen/structure to halt movement and prevent drying of the lubricating jelly. For larger specimens, an alternative to the usual excavated slides is to use a glass or acrylic ring (ca. 3–10 mm) glued with silicone onto a regular glass slide for microscopy, as depicted in Fig 2D. If the specimen of interest is properly halted within the liquid medium, serial images for posterior montage (i.e., stacking) will be possible for almost any structure using appropriate equipment.

### Conflicts of interest

The authors declare no conflicts of interest.

### Acknowledgements

We are grateful to thank Maria Isabel P. Balbi for valuable suggestions on the dehydrating and advertising about the thermal shock during the procedures of the protocol, and to Bryan N. Danforth for advice on preparing the slides shown in Fig. 2D. We are

thankful to two anonymous reviewers who provided useful suggestions to the manuscript. This project was partly supported by grant # 2011/09477-9, São Paulo Research Foundation (FAPESP) to E.A.B. Almeida, and by the fellowships numbers 2012/22261-8 and 2014/10090-0 to D.S. Porto also by FAPESP.

### References

- Ahn, K.-J., Ashe, J.S., 1996. Phylogeny of the intertidal aleocharine tribe *Liparoccephalini* (Coleoptera: Staphylinidae). *Syst. Entomol.* 21, 99–114.
- Alexander, B.A., Michener, C.D., 1995. Phylogenetic studies of the families of short-tongued bees (Hymenoptera: Apoidea). *Univ. Kans. Sci. Bull.* 55, 377–424.
- Ashe, J.S., 1992. Phylogeny and revision of genera of the subtribe *Bolitocharina* (Coleoptera: Staphylinidae: Aleocharinae). *Univ. Kans. Sci. Bull.* 54, 335–406.
- Beutel, R.G., Friedrich, F., 2008. A renaissance of insect morphology –  $\mu$ -Ct and other innovative techniques. *DGAE-Nachrichten* 22, 5–8.
- Deans, A.R., Mikó, I., Wipfler, B., Friedrich, F., 2012. Evolutionary phenomics and the emerging enlightenment of arthropod systematics. *Invertebr. Syst.* 26, 323–330.
- Hanley, R.S., Ashe, J.S., 2003. Techniques for dissecting adult aleocharine beetles (Coleoptera). *Bull. Entomol. Res.* 93, 11–18.
- Melo, G.A.R., 1999. Phylogenetic relationships and classification of the major lineages of Apoidea (Hymenoptera), with emphasis on crabronid wasps. *Sci. Papers (Nat. Hist. Mus. Univ. Kans.)* 14, 1–55.
- Moure, J.S., 1942. *Abelhas de Salobra* (Hym. Apoidea). *Papeis Avulsos* 2, 291–321.
- Porto D.S., Vilhelmsen, L., Almeida, E.A.B., 2015. Comparative morphology of the mandibles and head structures of corbiculate bees (Hymenoptera: Apidae: Apini). *Syst. Entom.*, <http://dx.doi.org/10.1111/syen.12156>.
- Roig-Alsina, A., Michener, C.D., 1993. Studies of the phylogeny and classification of long-tongued bees (Hymenoptera: Apoidea). *Univ. Kans. Sci. Bull.* 55, 123–160.