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RESEARCH ARTICLE

Description of an ancient social bee trapped in amber using diagnostic radioentomology

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Abstract The application of non-invasive imaging technologies using X-radiation (diagnostic radioentomology, 'DR') is demonstrated for the study of amber-entombed social bees. Here, we examine the external and internal morphology of an Early Miocene (Burdigalian) stingless bee (Apinae: Meliponini) from the Dominican Republic using non-destructive X-ray microtomography analysis. The study permits the accurate reconstruction of features otherwise obscured or impossible to visualize without destroying the sample and allows diagnosis of the specimen as a new species, *Proplebeia adbita* Greco and Engel.

Keywords *Proplebeia* · Stingless bees · Diagnostic radioentomology · Miocene · Burdigalian · MicroCT · Amber

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Introduction

Bees are a diverse lineage and many are important pollinators, familiar to the entomologist and layman alike. They range from mostly solitary species, to facultatively social, to those living in eusocial colonies (Michener, 1974, 2007). The monophyly and higher classification of bees have been well established, and the relatively stable relationships among these broader groups are being refined continually through the application of morphological and molecular techniques. Current evidence supports the conclusion that bees evolved from among the apoid wasps at least 125 million years ago (Engel, 1996, 2000, 2001a, 2004, 2011; Ohl and Engel, 2007; Michener, 2007) and most classifications place them as the clade Anthophila, likely sister to the family Crabronidae (Ohl and Bleidorn, 2006; Engel, 2011). All extant species, apart from the necrophagous *hypogea* group of *Trigona* (Roubik, 1982; Camargo and Roubik, 1991; Noll et al., 1996; Noll, 1997; Mateus and Noll, 2004; Camargo, 2008) and the tiny species of the genus *Lisotrigona* (Bänziger et al., 2009; Bänziger and Bänziger, 2010) feed their immatures a mixture of pollen and nectar (Roubik 1989; Michener, 2007; Engel, 2011).

Pollen and/or floral oil collection and transport by adults were made possible by a suite of behavioral and morphological adaptations, novelties that contributed to the rapid diversification of the angiosperms in the Cretaceous period (Thorp, 1979; Soltis et al., 2003; Grimaldi and Engel, 2005).

The bees have several characteristic morphological attributes such as branched or plumose body setae and broadened metabasitarsi (Michener, 2007; Engel, 2011). The highly eusocial stingless bees comprise the tribe Meliponini among the corbiculate Apinae (e.g., Engel, 2001b, 2005; Cardinal and Packer, 2007; Michener, 2007). In addition to extensive morphological and molecular data (e.g., Cardinal and Packer, 2007; Kawakita et al., 2008), the monophyly of corbiculate apines has been supported by studies investigating their internal anatomy. For example, Serrão (2001) noted that the proventricular morphology of Euglossini and Bombini consists of long columnar plates, triangular apices in Apini, while the Meliponini have slender and elongated plates. Accordingly, the proventriculus can be used as an important diagnostic structure for bee taxonomy (Greco et al., 2008), among a suite of other internal anatomical features (Engel, 2011). The examination of such characters often requires considerable manipulation, dissection, sectioning or even complete destruction of the specimen. Thus, the practical application of such data is at times hampered by the methods employed.

Traditionally, the morphological classification of bees has been conducted with the aid of dissecting microscopes which use light. The technique is understandably limited when used for amber inclusions (e.g., Wille and Chandler, 1964; Michener, 1982; Engel, 1995, 1996, 1997; Rozen, 1996; Camargo et al., 2000; Oliveira, 2002; Hinojosa-Díaz and Engel, 2008), particularly with specimens preserved in opaque pieces (Lak et al., 2008, 2009). Schlüter and Stürmer (1982) attempted to address methods of examining insect inclusions in opaque amber pieces as well as supplement traditional light microscopic study of clear specimens. Those researchers and Gerling and Hermann (1978), Gerling et al. (1981) and Velthuis and Gerling (1983) produced traditional X-ray radiographs of live bees, which provided the first, albeit limited, steps toward enhanced visualization of cryptic bee behavior and fossil material. More recently, detailed information for the study of bees has been obtained with the use of scanning electron microscopy (SEM) (e.g., Serrão, 2001, 2005) and transmission electron microscopy (TEM) (e.g., Araujo et al., 2005). While SEM and TEM studies currently provide the highest level of detail, sample preparation is laborious and often invasive or outright destructive (e.g., Serrão, 2001). SEM and TEM can be used for the investigation of amber inclusions (e.g., Grimaldi et al., 1994; Engel, 2001a), but these methods are generally unsuitable because they require destruction of the study material.

X-ray computerized tomography (CT) has been adopted previously to visualize macroscopic characteristics of insects and their behavior (Tollner, 1991; Fuchs et al., 2004; Greco et al., 2005, 2006, 2009; Perna et al., 2008). Conventional microfocus and synchrotron-based MicroCT is now emerging as a new method for the non-invasive imaging of insects at the microscopic level (e.g., Hörnschemeyer et al., 2002; Johnson et al., 2004, Wirkner et al., 2004; Tafforeau et al., 2006; Betz et al. 2007; Greco et al., 2008; Heethoff et al., 2009). The basic principles of conventional microfocus source MicroCT are similar to those used in medical CT scanners, and it is now possible to achieve spatial resolutions down to a few micrometers (Bettuzzi et al., 2004; Feeney et al., 2006). Diagnostic radioentomology (DR) is the collective term used for the non-invasive study of insects using X-radiation (radiographs, MacroCT and MicroCT). In this paper, we describe the internal and external morphology of an ancient social bee trapped in amber using non-invasive and non-destructive DR techniques.

Materials and methods

Sample and sample preparation

The sample selected for this study was collected from the La Bucara mine in the Dominican Republic (19°34′ N, 70°40′ W). The amber is a polished, semi-clear brown piece with many inclusions including a stingless bee at the thickest end (Fig. 1). The posterior of the bee's metasoma is at the extreme periphery of the piece's thick end, and the apices of both forewings have broken away from the sample over time. Age estimates of Dominican amber vary considerably in literature (e.g., Lambert et al., 1985; Grimaldi, 1995; Iturralde-Vinent and MacPhee, 1996). Nonetheless, most data indicate that the age of most Dominican amber, including the material in this study, is 16–19 Ma (Grimaldi, 1995; Camargo et al., 2000; Grimaldi and Engel, 2005). The sample had been polished prior to this study and thus required no extra preparation.

Light microscopy

For light microscopy, the bee was viewed using a Leica MZ12 stereomicroscope, Leica Microsystems GmbH Ernst-Leitz-Strasse 17–37 35578 Wetzlar. The Leica MZ12 has distortion-free 10× eyepieces with a resolution of 375 line-pairs per mm. Ideally, because of the thickness of the amber and air bubble inclusions and fractures present in the sample, it would have been better to cut the piece prior to light microscopy examination; however, the sample was intentionally preserved to enable visualization of the other biological inclusions using DR in future studies.

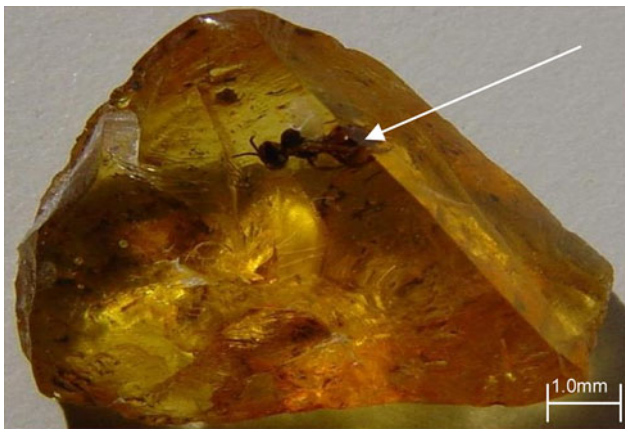

Fig. 1 Amber sample selected for study; a piece of polished, semi-clear, light brown amber from the Dominican Republic (Early Miocene: Burdigalian), with many inclusions. The stingless bee is at the widest end (*arrow*)

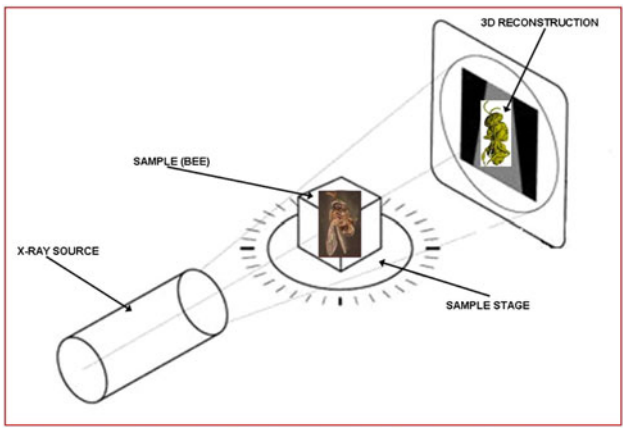

Fig. 2 A schematic diagram of sample positioning for DR. Essentially, the only preparation required is that the sample (bee) is positioned securely on the sample stage so that it remains motionless during the scan

DR-MicroCT

Scanning phase

MicroCT scans were performed using three systems: a commercial benchtop system, a custom designed scanner and the facility for MicroCT available at the SYRMEP beamline of the Elettra light source in Trieste (Italy). The first scans were performed at the Department of Clinical Research, University of Bern, using a MicroCT 40 system (by Scanco Medical AG, Brüttisellen, Switzerland). Prior to scanning, the sample was placed in a 20.5 mm cylindrical sample holder between the X-ray source and the CCD detector (Fig. 2). This sample positioning procedure for the scanning phase of a DR examination is the same for desktop and Beamline scanning.

The following Scanco scanning parameters were used:

- Tube operating conditions: HV peak was set at 45 kV and current was 177 μA
- High resolution mode (1,000 Projections/180°)
- Image matrix of 2,048 × 2,048 pixels
- Isotropic voxel size: 10 μm
- Integration time: 3 s
- Total number of 610 slices
- Measurement time: 10.5 h

Scans were converted to axial orientation with the Scanco software and 998 bitmap images (16 bit grayscale) were stored for 2D viewing and 3D rendering as a 983 Mb dataset.

The other scans were performed at Elettra, the third-generation synchrotron light source in Trieste (Italy) using two facilities for MicroCT, a conventional benchtop system (TOMOLAB) based on a microfocus generator and the MicroCT system available at the SYRMEP beamline (Abrami et al., 2005). The Elettra facilities are complementary in their use. While the beamline setup advantages include characteristics such as high spatial coherence, X-ray monochromaticity and parallel beam geometry, it is limited in the energy range as well as in the vertical beam dimension. TOMOLAB's advantages are its polychromatic spectrum peaked at high X-ray energies and high magnification due to the cone beam geometry, which are suited for studying larger samples and/or samples with higher absorption. TOMOLAB scans were performed using the following scanning parameters:

- Tube operating conditions: HV peak was set at 40 kV and current was 200 μA
- Source-to-sample distance: 12 cm
- Source-to-detector distance: 36 cm
- Isotropic voxel size: 8 μ
- Exposure time: 2.7 s
- Number of projections: 2,400 over 360°
- Measurement time: 1:48 h

SYRMEP beamline scans were performed using the following scanning parameters:

- X-ray energy: 15 keV
- Sample-to-detector distance: 20 cm
- Number of projections (over 180°): 1800
- Isotropic voxel size: 9 μ
- Exposure time: 0.9 s
- Measurement time: 1:48 h

The height of the beam was about 4 mm and because the sample thickness was approximately 7 mm, two scans were needed to cover the entire sample.

The above methods for the scanning phase of DR are usually performed by staff at the MicroCT facilities. Entomologists who would prefer to perform the scans can be

trained just as one is trained for the use of SEM or TEM equipment.

Image processing

Using multi-planar reformatting (MPR) algorithms, the 2D bitmaps were reformatted into a 3D model. The 3D reconstruction and analyses of the 2D bitmaps were performed as in Greco et al. (2005). The sample was further manipulated by adjusting window levels (WL) and window widths (WW) to enhance visualization of the morphological structures. Greatest visual enhancement was achieved at WL 110 and WW 35. The narrow WW reflected the closeness of the amber density to that of the bee's cuticle. For greater flexibility in software usage for image processing, the commercial VGSTUDIO MAX 2.0 voxel data analysis and visualization software (Volume Graphics GmbH, Wieblinger Weg 92a, 69123 Heidelberg, Germany) was used, while at the scanner workstations, Bee View 3D rendering software (DISECT Systems Ltd., Suffolk, UK) was used remotely on an office PC and laptop computer. The sample was viewed from many angles along randomly selected axes. Sections of the model were also removed along cutting planes, which were positioned by using the computer mouse. The cutting planes acted like a virtual scalpel enabling visualization of the bee's internal morphology without damaging the amber or its inclusions. Image magnification was performed when greater detail was required. Phylogenetic characteristics and morphological measurements were assessed with Bee View volume algorithms and on-screen linear callipers.

Results

Light microscopy

The color of the bee was brown to dark brown; however, it is possible that the bee was black when alive and that the cuticular melanin was altered over time. Indeed, many amber inclusions may appear cleared as a result of diagenesis, which somewhat lightens the cuticle. Moreover, teneral adult stingless bees are often lighter in coloration and so the more brownish color of the specimen cannot be considered diagnostic. Gross external morphological features of the bee such as the flagellomeres, coxae, trochanter and tibiae were visible to about the level of the mesothorax. The air bubbles, fractures and general thickness of the amber piece prevented adequate visualization of the more posterior morphology, including a lack of detail of the wings. Increasing light intensity created image degradation due to light diffracting from cracks, air bubbles and generalized opacity of the amber. Decreasing light intensity made it difficult to optically visualize the bee's morphological features (Fig. 3).

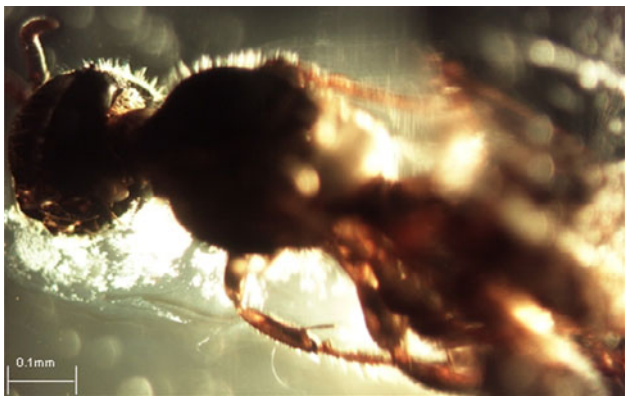

Fig. 3 Air bubbles, fractures and general thickness of the amber prevent adequate visualization of the metasoma, posterior mesosoma and wings. Image taken under optimal optical conditions (increasing or decreasing light intensity further degraded image quality)

DR-MicroCT

Although the resolution of MicroCT is not as fine as SEM or TEM and color cannot be discerned as with light microscopy, the images presented here demonstrate that DR was useful for viewing and assessing the external and internal morphology of amber inclusions accurately and for non-invasively identifying diagnostic morphological features (see also Pohl et al., 2010). DR is particularly useful for studying inclusions in opaque amber (Lak et al., 2008, 2009).

Gross external morphological features of the bee such as the flagellomeres, the articulations of the coxae, trochanters, tibiae, and tarsi, including the corbiculae of the metatibiae and the broadened metabasitarsi, were well visualized in the 3D reconstructions (Fig. 4). In addition, gross internal structures, such as the brain (including details of its anatomical regions), direct and indirect flight muscles and a loaded rectum were accurately represented (Fig. 5). Considering the specimen's age (16–19 Ma), the brain of this bee was particularly well preserved. The optic and antennal lobes were well reconstructed along with the dense central body and the protocerebral lobes (Fig. 6). The retinal zone was also well preserved. Adhesion of the retinal zone to the proximal surface of the compound eyes and the corresponding region on the distal surface of the medullae was evidenced by a thin, dense film of tissue (Fig. 6).

Taxonomy

A brief diagnosis is provided for the new species recognized during this work. The species matches the generic diagnosis of *Proplebeia* as it is understood based on the revision of

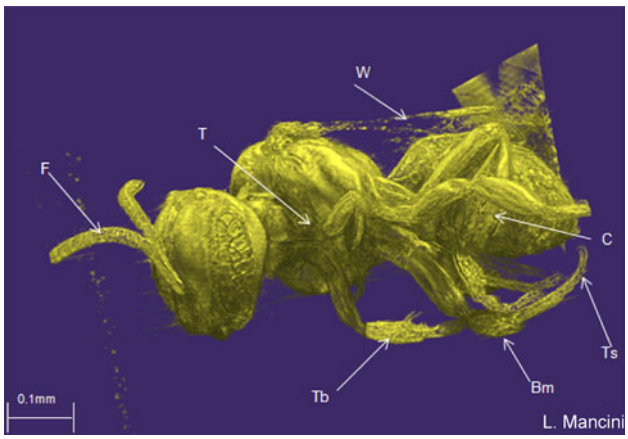

Fig. 4 Volume rendering image of the holotype worker of *Proplebeia abdita* Greco and Engel n. sp. in Early Miocene (Burdigalian) Dominican amber. Wings (*W*), flagellomeres (*F*), base of trochanter (*T*), tibiae (*Tb*), tarsi (*Ts*), the corbicula (*C*) of the metatibia and the broadened metabasitarsi (*Bm*) are all well visualized (TOMOLAB–VGSTUDIO MAX 2.0 rendering)

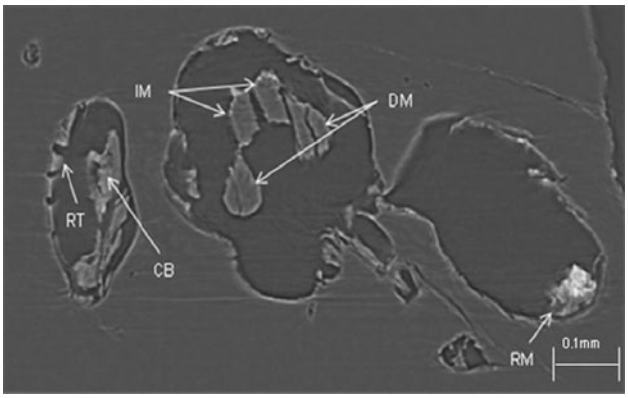

Fig. 5 Sagittal view of *Proplebeia abdita* Greco and Engel n. sp. holotype. Gross internal structures such as the central body of the brain (*CB*), retinal zone of the compound eyes (*RT*), direct (*DM*) and indirect (*IM*) flight muscles and a loaded rectum (*RM*) were accurately visualized (SYRMEP-Bee View rendering)

Camargo et al. (2000), an extinct genus of the stingless bee lineage which itself most likely had West Gondwanan origins (Michener, 2007; Rasmussen and Cameron, 2010). Morphological terminology follows that of Camargo et al. (2000), Engel (2001a), and Michener (2007), while the format for the diagnosis generally follows those features highlighted by Camargo et al. (2000) across species of *Proplebeia*. The species generally matches the description of the worker for *P. dominicana* (Wille and Chandler) provided by Camargo et al. (2000) except in those features discussed. Although the characters of the vestigial sting apparatus are of some use in Meliponine systematics (e.g., Michener, 1990), these sclerites could not be imaged relative to the surrounding amorphous and likely dessicated metasomal tissues.

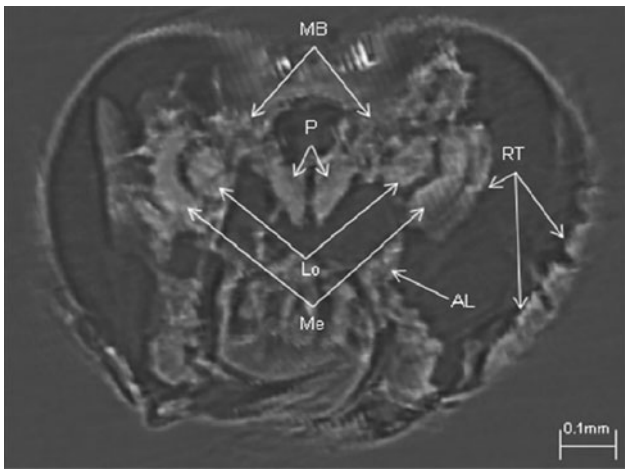

Fig. 6 An axial view of the bee's head capsule. The brain of this bee was particularly well preserved as evidenced by the optic lobes including the medullae (*Me*) and lobulae (*Lo*), antennal lobes (*AL*), protocerebral lobes (*P*) and the mushroom bodies (*MB*). The retinal zone (*RT*) was also well preserved (Scanco-Bee View rendering)

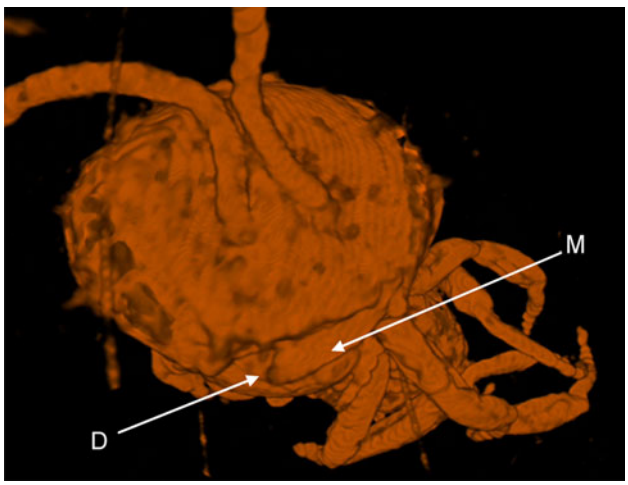

Fig. 7 A 3D reconstruction of the bee showing details of the tridentate mandible (*M*) with a slight emargination separating the three denticles (*D*)

Proplebeia abdita Greco and Engel, new species

Diagnosis: The worker of the new species can be distinguished from those of other species of *Proplebeia* by the tridentate mandible (Fig. 7), with a slight emargination separating the three denticles (bidentate in all other *Proplebeia*), the presence of only five to six spine-like setae comprising the rastellum (7–9 in all other *Proplebeia*) and the relatively straight inner compound eye margins (slightly concave in other *Proplebeia*). Among the species of the genus, *P. abdita* is most similar to *P. dominicana,* but in addition to the aforementioned attributes, can be separated by second flagellomere slightly longer than the third

(slightly shorter in *P. dominicana*) and the larger body size (ca. 3.8 mm in *P. abdita*, ca. 3.0 mm in *P. dominicana*). In the larger size, the new species more closely approximates *P. vetusta* (3.8–4.4 mm; forewing 2.88–3.16 mm), but again the above attributes can separate the two species. In addition, *P. abdita* lacks the long, straight setae of the metasomal sterna that are present in *P. tantilla*. Moroever, *P. tantilla* is a considerably smaller species, the smallest for the genus (ca. 2.1 mm in length; forewing length, 1.96 mm).

Metrics: Total body length, 3.79 mm; forewing length, 2.75 mm; width, 1.22 mm; head width, 1.36 mm, length, 1.25 mm; compound eye width, 0.33 mm, length, 0.81 mm; metasoma width, 1.03 mm.

Holotype: Female (worker); early miocene (Burdigalian), dominican amber; deposited in the Graptolites and Fossil Arthropods collection of the Natural History Museum, London (accession number: NHM II 3044).

Etymology: The specific epithet is the Latin term *abditum*, meaning "concealed" or "hidden".

Discussion

Diagnostic radioentomology permitted the comprehensive examination of this ancient specimen, where other methods were (in the case of light microscopy) and would be (in the case of SEM or TEM) found to be less reliable or unsuitable because of their destructive nature. We were able to accurately assess the bee's anatomical characteristics with Bee View volume visualization algorithms and perform precise morphometric measurements with the program's on-screen linear calipers. As a result, we were able to produce details of a previously undescribed species, *P. abdita* Greco and Engel. This study demonstrated that all three methods were appropriate for visualizing the specimen. Thus, entomologists can consider which facility would provide the best option for them. In addition to the application of DR to this particular bee, its more extensive use on historical type material (e.g., the holotype of *P. dominicana*, other amber-preserved bees or even unique specimens of rare modern species) will permit a more complete characterization of these taxa and comprehensive comparisons between them and their modern counterparts. Improved anatomical understanding of these taxa will greatly enhance phylogenetic reconstructions utilizing paleontological data and potentially revise our paleoecological perspectives of early pollinators. It is hoped that by highlighting the utility of DR for characterizing an ancient social bee that these techniques might be more broadly applied to social bee biology and anatomy, much in the tradition of Gerling et al. (1981) earlier applications of novel imaging methods and in the way it has been applied to the study of termites and living stingless bees (e.g., Fuchs et al., 2004; Greco et al., 2005), as well as solitary bee species (e.g., Greco et al., 2006, 2008).

Acknowledgments This work is dedicated to the memory of Prof. J.M.F. Camargo, leading authority on the systematics of stingless bees and who had initially participated in this work, suggesting that the specimen belonged to a new species. We regret his untimely passing. The authors would like also to thank Giuliana Tromba, Lucia Mancini and Nicola Sodini for their contribution to the experimental work. We are grateful to DISECT Systems Ltd for donating their 3D rendering and telelinking software for this study, and to the two anonymous reviewers for their positive feedback on an earlier draft of the manuscript.

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