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Paleontological Contributions

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EXTRACTION OF INCLUSIONS FROM (SUB)FOSSIL RESINS, WITH DESCRIPTION OF A NEW SPECIES OF STINGLESS BEE (HYMENOPTERA: APIDAE: MELIPONINI) IN QUATERNARY COLOMBIAN COPAL

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ABSTRACT

Amber and copal are renowned for preserving insects and other inclusions with lifelike fidelity. However, due to their frozen-in-time nature, they are taxonomically subequal to Recent insects, which commonly require dissection in order to identify them to species level. This can be overcome to a degree through digital dissection using computed tomography, but this technique is time consuming, expensive, and not widely accessible. We attempted to dissolve inclusions out of Dominican and Baltic ambers and Quaternary Colombian copal using chloroform. Extraction of specimens from amber was unsuccessful, but we were able to extract a stingless bee from the less polymerized copal and dissect it under a microscope as if it were a recently caught insect. We were able to examine all of the features that are considered to be diagnostic for extant species, and thus our subfossil is taxonomically equivalent to a living species. The copal bee is a new species of the *Trigonisca longitarsis* species group (=*Dolichotrigona*), which is described and figured herein as *Trigonisca ameliae* n. sp. (Hymenoptera: Apidae). The ability to extract inclusions from (sub)fossil resins facilitates more accurate studies of Quaternary tropical forest biodiversity, in addition to molecular paleobiology and taphonomic physiochemical changes resulting from diagenetic processes following entombment in copal- and amber-forming resins. Colombian copal is radiocarbon dated within the age range <60 (postbomb) to 10,612 ± 62 years old.

Keywords: amber, chloroform extraction, entomology, fossil resin, insect, neotropical, paleontology

INTRODUCTION

The nomenclature of (sub)fossil resins is problematic (Vavra, 2009), and the distinction between amber and its subfossilized precursor, copal, has not been clearly defined. Generally speaking, copal is much younger and less polymerized (and hence softer) than amber. Many paleontologists consider copal too young to be of interest and, as a result, little research has focused on this material. However, Penney and Preziosi (2010, 2013), Penney and others (2012b), and Penney and Green (2012) highlighted the potential value (at many different levels) of subfossils in copal.

Both amber and copal are renowned for preserving insects and other inclusions with lifelike fidelity (Penney, 2010), including, in some cases, at the subcellular level (Koller, Schmitt, & Tischendorf, 2005). However, the exact mode of preservation in amber is unknown, although it is commonly referred to as a kind of mummification process resulting from rapid fixation and dehydration of anything that became trapped in the original resin secretion. The process will presumably vary, albeit possibly only slightly, in different ambers as the result of differences in the chemistry of the resin secreted from the amber producing trees, which originated from various different families (e.g., Langenheim, 1995). Certainly, the process is not uniform for all inclusions. For example, the recent application of X-ray computed tomography in studies of fossils in amber has demonstrated that, in some instances, internal organs are preserved, e.g., in the case of a strepsipteran preserved in Eocene Baltic amber (Pohl & others, 2010), whereas digital dissection of a spider preserved in Eocene French amber revealed that nothing substantial was preserved internally (Penney & others, 2007).

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Although the application of computed tomography and synchrotron scanning to amber and copal inclusions has aided the study of these fossils considerably by narrowing the divide between paleontological and neontological taxonomy (Penney & others, 2007, 2011, 2012a, 2012d; Bosselaers & others, 2010; Pohl & others, 2010; Soriano & others, 2010; Dunlop & others, 2011, 2012) and by allowing us to study unique paleoethology (Dunlop & others, 2012; Penney & others, 2012c), they are still considered by many neontologists to be taxonomically subequal to extant forms. These techniques are time consuming, very expensive, require specialist technical expertise, and are restricted in their availability. It would be greatly beneficial if fossils could be extracted from their (sub)fossilized resin matrix so that they could be studied alongside extant forms using the same techniques (including electron microscopy; e.g., Azar & others, 1999) and taxonomic characters for their identification. Extraction of fossils from (sub)fossilized resins would also facilitate studies of their molecular paleobiology and taphonomic physiochemical changes resulting from diagenetic processes, in addition to more accurate quantitative studies of Quaternary tropical forest biodiversity.

Previous successful studies to extract inclusions by dissolving amber include those of Azar (1997), Azar and others (1999), and Mazur and others (2012). Azar (1997) was able to dissolve upper Neocomian–basal lower Aptian (ca. 135 Ma) amber from Lebanon. Various solvents (e.g., ethanol, butanol, acetone, toluene) were tried, but only chloroform gave satisfactory results, yielding articulated but fragile fragments of insect cuticle, including heads, abdomens, wings, and genitalia. Mazur and others (2012) found xylene, toluene, chloroform, orange oil, and turpentine oil useful for dissolving inclusions out of Eocene (ca. 52 Ma) Cambay amber from India. In both, the aforementioned studies, the extracted inclusions were very fragile, no doubt because of their great antiquity. Here, we apply a similar technique to inclusions preserved in Dominican amber, Baltic amber, and Colombian copal, and describe a new species of stingless bee from the last deposit.

Stingless bees (tribe Meliponini) are one of only two highly eusocial bees, the other being the well-studied honeybee (tribe Apini). Unlike Apini, with only 11 species in the single genus *Apis*, stingless bees form a large and diverse taxon that consists of 60 genera, many of which are poorly known (Rasmussen & Cameron, 2010). They are found in abundance in warm humid forests around the globe and have left an imprint in the fossil record spanning most of the Cenozoic. Poinar (1999) proposed that the demise of stingless bees known from Dominican amber but absent from the Greater Antilles today resulted from a cool period associated with increased aridity during the Plio-Pleistocene, although Peñalver and Grimaldi (2006) suggested that the insularization of Hispaniola was probably a more important factor. Hence, fossils and subfossils of this group of organisms have the potential to be informative about past biogeographical processes and the comparative extinction resistance of island versus continental lineages. The relatively young age of many copals means that inclusions may belong to extant species, even though they may have not yet been described in the scientific literature. The possibility that a copal inclusion may belong either to an extant or an extinct species highlights the importance of considering both neontological and paleontological data when describing new taxa from copal-producing regions (Penney, Ono, & Selden, 2005; Azar, Nel, & Waller, 2009).

MATERIALS AND METHODS

Our samples included one specimen of Miocene Dominican amber (ca. 16 Ma) containing a flat-footed beetle (Coleoptera), one specimen of Baltic amber (ca. 44–49 Ma) containing a small fly (Diptera), and a specimen of sub-Recent Colombian copal (post-WWII) (radiocarbon dated at the University of Arizona AMS facility) containing stingless bees (Apidae: Meliponini: *Trigonisca* sp.) (Fig. 1.1). A second Colombian copal specimen, also containing the same species of stingless bee, was sent for dating at the Arizona AMS facility. This came back with an age of 10,612 ± 62 years, representing the oldest formally dated Colombian copal sample. The specimens were trimmed to a small workable size using a High-Tech diamond trim saw, and then each specimen was further shaved down using a scalpel into a small cube of approximately 0.4 g (~4 mm^3), with the inclusion situated in the middle. Microphotographs were assembled from a stacked series of digital images recorded by a Nikon Coolpix 4500 camera mounted on a Leica M10 stereomicroscope with 0.63× and 1.6× planapochromatic objectives (Green, 2005).

Using a laminar flow hood, each specimen was placed with 5 ml of chloroform into a glass tube with a screw-top lid, and then placed in a water bath (with the rocking motion switched off) at 40° C and left for 48 hours, during which time they were checked periodically.

RESULTS

After 48 hours, the copal sample appeared to have dissolved to a greater extent than either of the amber samples. However, there was a highly viscous, nondissolved fraction floating across the surface of the chloroform. The inclusion had dissolved out fully intact and was positioned immediately below this fraction. When the tube was shaken gently, the inclusion floated freely in the liquid below the viscous layer. For all intents and purposes, it resembled a Recent entomological specimen preserved in alcohol. The amber samples had dissolved to a degree, but in a much less consistent manner. Some parts had softened and come apart to form separate gooey masses, and there were also small, hard fragments of nondissolved amber. The insect inclusions had disintegrated, not unexpectedly, leaving only tiny black fragments as evidence of their previous existence. Leaving the specimens in the water bath for several days longer did not result in any significant changes in the amber samples. Thus, the amber samples were set aside as unworkable in terms of their taxonomic value, whereas the bee was successfully extracted from the copal and identified as a new species, following dissection in alcohol under a stereomicroscope.

SYSTEMATIC PALEONTOLOGY

APIDAE Latreille, 1802
APINAE Latreille, 1802
TRIGONISCA Moure, 1950
LONGITARSIS species group

Type species.—*Melipona longitarsis* Ducke, 1916, p. 82, 88, 90, fig. 25a.

Figure 1. *Trigonisca ameliae* Penney n. sp. in Quaternary copal from Colombia: *1,* holotype (NHM II 3059 [1]), scale bar = 1.0 mm; *2,* paratype 1 (NHM II 3059 [2]) showing wing venation; *3,* paratype 2 (NHM II 3059 [3]); *4,* close-up of holotype; *5,* close-up of holotype showing lack of setae on head and antennae; *6,* paratype 3 showing left mandible dissected from the head after extraction of the inclusion using chloroform (with trace outline of *T. moratoi* mandible for comparison) (new).

Included species.—*Trigonisca browni* (Camargo & Pedro, 2005), *T. chachapoya* (Camargo & Pedro, 2005), *T. clavicornis* (Camargo & Pedro, 2005), *T. longitarsis* (Ducke, 1916), *T. martinezi* (Brèthes, 1920), *T. mendersoni* (Camargo & Pedro, 2005), *T. moratoi* (Camargo & Pedro, 2005), *T. rondoni* (Camargo & Pedro, 2005), *T. schulthessi* (Friese, 1900), *T. tavaresi* (Camargo & Pedro, 2005).

Comments.—The above taxa were described or listed under *Dolichotrigona* by Camargo and Pedro (2005). However, the most widely accepted classification for Meliponini worldwide is that of Michener (2007), in which *Dolichotrigona* forms the *longitarsis* species group of *Trigonisca*. In order to enforce monophyletic taxa within Meliponini, Rasmussen and Cameron (2010) confirmed that *Dolichotrigona* should be synonymized under *Trigonisca*, *sensu* Camargo and Pedro (2005). To facilitate comparison, the following diagnosis and description are based on the characters used in the genus revision by Camargo and Pedro (2005).

TRIGONISCA AMELIAE Penney n. sp.

Figures 1–2

Etymology.—The specific epithet is a matronym after Amelia Jan Penney, daughter of the first author.

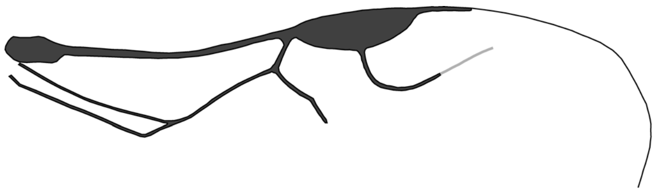

Figure 2. Wing venation of *Trigonisca ameliae* Penney n. sp. in Quaternary copal from Colombia, as drawn from paratype 1 (NHM II 3059 [2]) (see Fig. 1.2) (new).

Material.—The specimens are held in the collection of the Natural History Museum, London, United Kingdom (repository number: NHM II 3059). Holotype worker female (NHM II 3059 [1]) and two paratype workers (NHM II 3059 [2] and [3]) all preserved in the same piece of copal. A third paratype from the same piece of copal was dissolved out for dissection. The copal piece has one Diptera (Phoridae) and one Hymenoptera (Formicidae) as syninclusions.

Diagnosis.—Mandible with two teeth, anterior tooth distinctly larger, and the angle between the teeth sharp and approximately 90 degrees; head and antennae lacking setae and spines.

Dimensions (in mm).—Approximate total length, 2.7; head, 0.6; thorax, 0.9; abdomen, 1.2; maximum width of head, 1.1; tibia III, 0.9; anterior wing, 2.2.

Color.—Head, thorax, and abdomen dark red-brown, appearing almost black in some specimens (Fig. 1.1–1.5), but also copper colored under reflected light. There is no evidence of any other color patches (although sometimes these are not preserved in copal and amber specimens). Mandible yellowish (Fig. 1.6). Scape yellow basally and for most of its length, becoming suffused with black distally to appear brownish; pedicel and flagellar segments similarly brownish. Proximal leg segments dark reddish brown, basitarsus appears yellowish in some specimens under reflected light; mediotarsus, distitarsus, claws, and arolium yellow. Wing membrane hyaline; forewing pterostigma with dark margins, veins and inner area of pterostigma pale yellowish brown. Note: these colors and patterns may be the result of diagenetic processes, and thus may not be representative of how living specimens of this species may have looked. Nonetheless, somatic patterns are often readily observable in copal inclusions.

Pubescence.—Antennae, head, and thorax lacking setae and spines (Fig. 1.5); legs with inconspicuous pale setae present on tibiae and tarsal segments.

Integument.—Very finely punctate.

Shape and proportions.—Head 1.5× wider than long, eyes 2.25× longer than wide (Fig. 1.5). Mandibles with two teeth, anterior tooth distinctly larger, and angle between teeth sharp and approximately 90 degrees (Fig. 1.6). Scape approximately 10× longer than wide. First flagellomere subconical and wider than long, remaining flagellomeres cylindrical and slightly longer than wide, with distalmost twice as long as wide; lacking setae but with small, whitish, scalelike sensillae. Lateral ocelli slightly closer to median ocellus than to compound eyes. Interocellar distance equal to diameter of median ocellus. Tibia III (Fig. 1.4) approximately three times longer than at its greatest width (distally), forming an elongate triangle with posterior distal extremity drawn out to a point. Basitarsus III two times longer than broad; distal posterior corner angle acute. Wing dimensions cannot be measured accurately due to folding, but venation and pterostigma appear typical for genus (Fig. 1.2, Fig. 2).

Comments.—Based on the morphological characters used for delimiting the separate species in the *longitarsis* (*Dolichotrigona*) species group used in the revision of Camargo and Pedro (2005), *Trigonisca ameliae* sp. nov. can be considered intermediate between *T. moratoi* (with regard to mandibular armature) and *T. mendersoni* (with regard to the setae on the head and antennae). The mandible is very similar to that in the former species but differs in the position of the minor tooth, which is located more distally in the new species (Fig. 1.6). The head and antennae lack setae and spines, very similar, but not identical, to the latter species, which has a few setae on the antenna (Camargo & Pedro, 2005, fig. 17) and also in the ocellar region (Camargo & Pedro, 2005, fig. 25). This absence is highly unlikely to be an artifact of preservation, as setae do occur elsewhere on the inclusions and even ultrafine modified setae, such as trichobothria are preserved in Cretaceous fossil resins more than 100 million years old (e.g., Penney, 2006).

Trigonisca moratoi and *T. mendersoni* occur in close proximity to one another. *T. mendersoni* is known only from Brazil, the Rio Purus region of Ipixuna, to the western end of Rondônia and East of Acre, whereas *T. moratoi* is known only from the states of Amazonas and Acre, Brazil (Camargo & Pedro, 2005). Hence, they occur at the same latitude and are separated by only eight degrees of longitude, based on the very few known records. Only a handful of specimens were examined during the description of these two species, so the extent of any intraspecific, geographic variation cannot be determined. Thus, we cannot rule out the possibility that these two species are synonymous with one another and also with our new species. Also, mandibles may become worn in older individuals (C. Rasmussen, personal communication, 2012), and thus may not be the most useful character for species diagnosis. However, based on the current taxonomic status of this group, which was revised by very experienced researchers, we believe that erecting a new species for our specimens is the best option available. A future study based on larger sample sizes of broader geographical coverage, possibly including molecular techniques and the study of male genitalia, would be useful to confirm the validity of the currently accepted species.

Despite the revision of Camargo and Pedro (2005), the extant Neotropical stingless bees belonging to this group are poorly known. Evidence for this lies in the number of new species described in the revision and also in the limited distribution and ecology-habitat data presented. However, stingless bees have been reasonably well studied in New World ambers (Willie, 1959; Willie & Chandler, 1964; Camargo, Moure, & Roubik, 1988; Camargo, Grimaldi, & Pedro, 2000; Greco & others, 2011), so it is surprising that no current experts in this group have examined specimens in Colombian copal. A stingless bee in copal was described by Moure and Camargo (1978), although the provenance of the material was unknown. The inclusion showed affinities to the extant Afrotropical fauna, and so it most probably originated from Madagascar, a well-known source of fossiliferous copal. Although our new species is known only from subfossils in copal that originate from Santander, Colombia, the possibility that it is an extant species that still occurs in South America cannot be entirely ruled out. The only extant species recorded from Colombia is *Trigonisca schulthessi* (Friese), which differs from the copal specimens by possessing a single, small mandibular tooth and short setae on the antennae (Camargo & Pedro, 2005).

DISCUSSION

Given that the attempts to dissolve specimens out of amber resulted in total destruction of the inclusion beyond recognition, with only particulate fragments remaining, we do not recommend this approach. Our studied amber inclusions were no doubt typical of most fossil arthropods preserved in amber in consisting of only hollow spaces lined with a thin layer of diagenetically altered cuticle. However, our findings contrast with those of Azar (1997), who was able to recover articulated elements of fossilized insect bodies from Lebanese amber. Maybe this is due to differences in the resin chemistry or maybe Azar (1997) used much thinner samples than we did. It should be noted that Cambay amber from India derives from a dammar-like resin, and so is only weakly polymerized and cross-linked (Rust & others, 2010), compared to the majority of other ambers of Cenozoic age (e.g., Baltic and Dominican), so the fact that Mazur and others (2012) were able to dissolve it in organic solvents is not particularly unexpected. An alternative method of accessing (but not completely extracting) inclusions fossilized in amber is to crack the amber open using liquid nitrogen, as employed by Stankiewicz and others (1998), or by cutting the specimen to a small size, cutting a groove around the inclusion and then gently splitting the amber apart (Grimaldi & others, 1994).

However, the ability to extract inclusions from copal is potentially useful for various areas of paleobiological research. Unfortunately, in the extraction of the bee from the chloroform it was necessary to pull it through the viscous nondissolved fraction, and it became coated with the sticky residue, which started to harden and became difficult to work with as it cooled in air. Various attempts were made to remove this, such as washing it with absolute alcohol as an alternative solvent, also in a hot water bath, but without success. A second specimen from the same piece of copal kept in a water bath at 40° C for six days appeared to have dissolved completely, and the bee sank to the bottom of the tube when it was shaken slightly. There was barely any sticky film over the fluid surface, and it was possible to pipette the inclusion out of the fluid without any sticky coating. This specimen was extracted in a dedicated ancient DNA clean room for destructive DNA sampling, so it was not possible to take any images of it. The ability to fully dissolve the resin would be highly beneficial for future studies, and subsequent research by resin chemists would be particularly welcomed.

Nonetheless, we were able to recover the stingless bee from the first sample, articulated and in its entirety, which could then be dissected as if it were a recently caught specimen, albeit with some hindrance from the sticky coating. We were able to examine important taxonomic features, such as the mandibular dentition, that were impossible to see in the individuals preserved inside the copal matrix, resulting in the new species described herein. It is worth noting that the extracted specimen seemed to be a little more fragile than might be expected in recently preserved extant material, and so such specimens should be treated with particular care. At this stage, the long-term fate of such extracted specimens is unknown, but we would expect them to survive reasonably well if preserved in alcohol.

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