9. Pollen analysis of geopropolis and propolis from stingless bees

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Abstract
The present study aims to review palynological analysis of geopropolis and propolis obtained from stingless bees in South America. Such studies are scarce and most analyzed samples are from Brazil, with a few from Bolivia and Venezuela. High diversity in pollen types, along with plant tissue fragments, hyphae, fungal spores, and amorphous organic matter were found. Sand or clay were always present. Pollen analysis of geopropolis helps to characterize vegetation surrounding the collection site and provides data corroborating physico-chemical analyses.
Key words: cerumen, geopropolis, Meliponini, pollen analysis, propolis, **s**tingless bees, vegetation

Introduction
The Meliponini are social bees that live in tropical and subtropical countries. They visit flowers of the native vegetation, and are considered generalists when harvesting pollen and nectar (Ramalho et al., 2007). Biesmeijer et al. (2005) point out that some species have more or less easy access to pollen and nectar of different flowers and that their productivity depends on skill in handling the floral parts, materials secreted or exuded by plants (lipophylic material on leaves, mucilages, gums, resins, trichomes, etc.). These materials are collected, enriched with salivary secretions, transported and used in architectural and biological functions of bee nests and colonies (Bankova et al., 2000). Although many are docile, stingless bees can be aggressive in competing for food resources with the non-native *Apis mellifera* L., reducing the available trophic resources (Roubik, 1978, 1989; Schaffer et al., 1983).

The product elaborated by stingless bees is named geopropolis when this bees use clay particles mixed with wax and resins (Nogueira-Neto, 1953). Using wax and resins only, the resulting product is named cerumen (Nogueira-Neto, 1953; Roubik, 1992). Studies are still scarce on characteristics of geopropolis. This mixture of resin exudates, originated from several plant sources, mixed with wax, silt and sandy fragments, differs from propolis of *Apis mellifera* due to the presence of clay particles and absence of plant trichomes (Barth and Luz 2003). Due to the amount of mineral content, some geopropolis samples may show less malleability when compared to propolis samples. Meliponini use geopropolis in order to prevent mechanical damages and to seal the hives, acting as a thermoregulatory agent, avoiding exposure to air currents and to colony infestation (Teixeira et al., 2003).

In the world's temperate zones the dominant propolis source is the *Populus* spp. Pollen analysis over the world were performed mainly on propolis from *A. mellifera*. They were carried out primarily by Ricciardelli D'Albore (1979) who analysed 56 samples from several countries and Warakomska and Maciejewicz (1992) from Polish regions. There are some studies on palynological analysis of geopropolis and propolis from South America, mainly collected in Brazil from different species of bees: *Apis* (Barth, 1998; Barth et al., 1999; Bastos, 2001; Barth and Luz, 2009; Luz et al., 2009; Freitas et al., 2010; Freitas et al., 2011); *Frieseomelita varia*, *Lestrimellita limao*, *Melipona quadrifasciata*, *Nannotrigona testaceicornis*, *Tetragonisca angustula*, and *Trigona recursa* (Barth, 2006), *Melipona grandis*, *Scaptotrigona depilis*, *Scaptotrigona polysticta* from Bolivia, *Melipona mondury*, *Melipona quadrifasciata* and *Tetragonisca angustula* from Brazil, and *Lestrimelitta limao*, *Melipona favosa*, *Scaptotrigona* sp. and *Tetragona clavipes*, from Venezuela (Freitas et al., 2012).

Pollen grains appear in geopropolis (Barth and Luz, 2003), besides other structured elements such as sand, clay, plant tissue fragments, hyphae and fungal spores, and also amorphous organic matter, as contaminants. After chemical treatment to remove organic debris and other content which obscures structural elements, the pollen spectra obtained from geopropolis residues may contain nectariferous, polleniferous and anemophilous pollen grains, similar to cerumen and propolis. Thus, pollen analysis is a valuable tool in determining the origin of geopropolis, propolis and cerumen, and useful in characterizing regional biota and particularly flora (Barth et al., 1999; Barth and Luz, 2003).

In this review, a brief summary on general composition, pharmacological activity and quality control of propolis is introduced to the reader before palynological methods and analysis are discussed in more detail for geopropolis produced by stingless bees.

9.1 Composition, pharmacological activity and quality control

Bud exudate of poplar (*Populus*) is the dominant source of propolis in many temperate regions whereas in the tropics other plant sources are involved (Barth and Luz, 2009; Bastos, 2001) Nevertheless, most propolis samples share considerable similarity in their overall chemical nature: 50% resin (composed of flavonoids and related phenolic acids), 30% wax, 10% essential oils, 5% pollen and 5% other organic compounds (Dobrowolski et al., 1991).

The biological and pharmacological activies of propolis elaborated by *Apis mellifera* has been widely studied (Marcucci, 1995; Kujumgiev et al., 1999). Polyphenols are active molecules of propolis with inhibitor roles of enzymes acting at hormone, neurotransmitter, and free radical scavenging levels (Havsteen, 2002). Propolis is regarded as an ancient remedy valid in modern medicine (Castaldo and Capasso, 2002), and also recommended to prevent diseases by maintaining or improving human health for its anti- microbial, antioxidative, anti-ulcer and anti-tumor activities (Loflty, 2006). Pharmacological studies of geopropolis shows its antibacterial and antioxidant activity and a great concentration of flavonoids also (Bankova and Popova, 2007; Dutra et al., 2008; Manrique and Santana, 2008), giving a support for quality control and beekeepers information.

Measurements of polyphenols in propolis need analytical extractions and quantifications, based on diverse techniques such as spectrophotometry, High Performance Liquid Chromatography (HPLC) with a Diode Array Detector (DAD), Capillary Electrophoresis (CE), Supercritical Fluid Extraction (SFE), Pressurized Liquid Extraction (PLE), Microwave-Assisted Extraction (MAE), Ultrasound-Assisted Extraction (UAE), Electrospray (ES) — constantly under scrutiny and improvement in bee products (Gómez-Caravaca et al., 2006; Pérez-Pérez et al., 2012). Recently, electrospray techniques were used for native Brazilian stingless bees (Sawaya et al., 2007), especially *Scaptotrigona* (Sawaya et al., 2009). Later, cytostatic action of *Scaptotrigona* sp. propolis (from Maranhão State, Brazil) alone and combined with temozolomide, was demonstrated in glioblastoma cells from tumor brain tumor (Borges et al., 2011). Gas-chromatography and spectrometry were used to fingerprint cerumen of the Australian stingless bee *Tetragonula carbonaria*, and 5-lipoxygenase (5-LOX) cell-free assays were applied to scan anti-inflammatory properties (Massaro et al., 2011). Propolis is a variable product due to its diverse botanical, geographic and entomological origin, therefore detailed analysis or some form of 'quality control' is needed to support medicinal use.

9.2 Palynological methods for geopropolis and propolis

The palynological processing of geopropolis and propolis samples followed standard methodology (Barth, 1998). About 0.5 g of scraped propolis was extracted overnight with ethanol. Next, the sediment was treated with KOH, ultrasound, and sieved to eliminate large fragments. At this stage, two microscope slides were prepared for inspection to detect organic residues that may be destroyed in subsequent chemical processing of the sample.

The acetolysis method (Erdtman, 1952) was then applied, and two additional samples on microscope slides were mounted using glycerin jelly (p.a.), one stained with basic fuchsin (p.a) and the other unstained. The target sum was 300 pollen grains or more per sample. The definition of pollen classes followed Zander (Louveaux et al., 1978), and was used for qualitative and quantitative analyses. Samples were observed using light and polarized light microscopy. Pollen types were identified using a Neotropical pollen atlas (Roubik and Moreno, 1991) and reference slides.

9.3 Palynological analysis of geopropolis and propolis
Studies concerning the palynological analysis of geopropolis and stingless bee propolis samples are scarce. Other stingless bee products are better studied, such as honey and pollen. Images of geopropolis and propolis palynological slides from Bolivia, Brazil and Venezuela are shown in Figure 1.

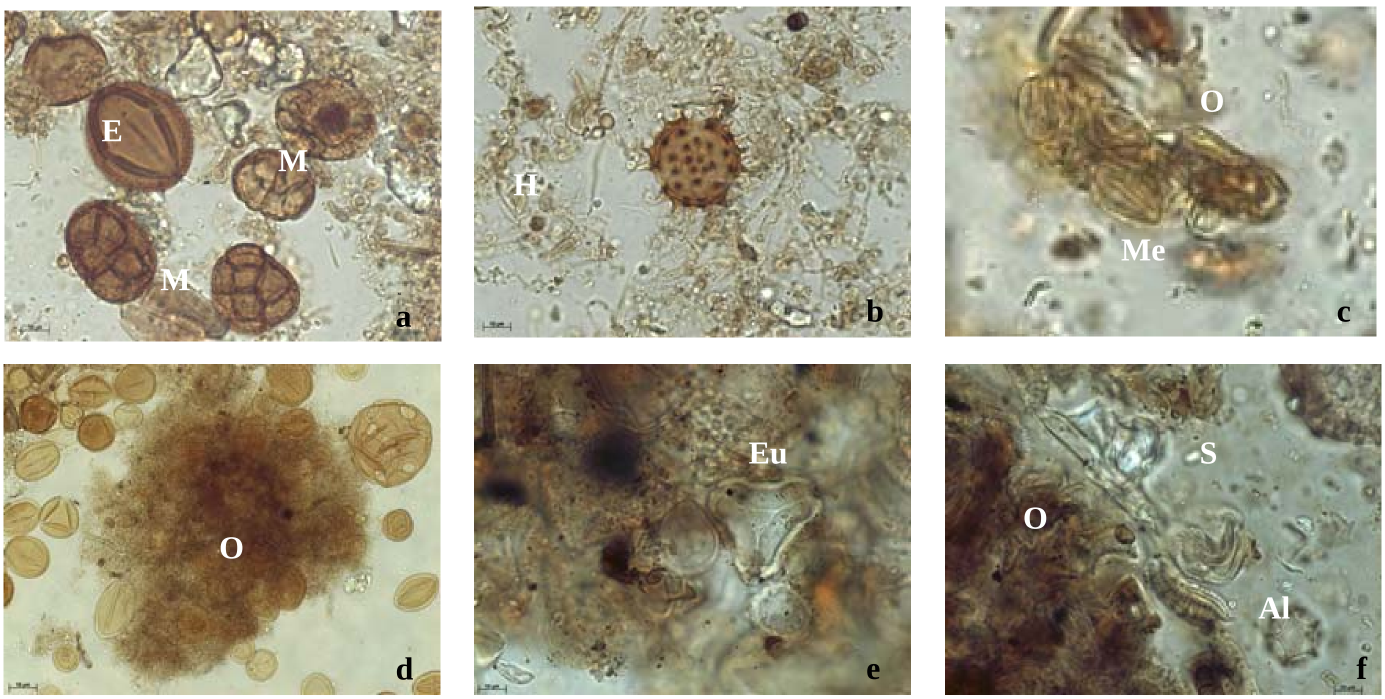

Figure 1. Geopropolis from Bolivia (a-b), Paraná, Brazil (c), Venezuela (d) Ribeirão Preto, Brazil (e-f)
a) polyads of Mimosaceae (M), a monad of Euphorbiaceae (E), and debris. b) pollen grain of Asteraceae (A) and many hyphae (H) and fungal spores. c) group of pollen grains of Melastomataceae (Me), organic material (O) and debris. d) several pollen types and a compact organic material (O). e) *Eucalyptus* (Eu) pollen grain inside organic debris. f) pollen grain of *Alternanthera*/Amaranthaceae (Al), sand crystals (S) and organic material (O).

A total of 10 geopropolis samples from the Brazilian states of Espirito Santo, Minas Gerais and São Paulo were analyzed by Barth and Luz (2003). They show dominant pollen types of *Eucalyptus* (Myrtaceae) in a sample of *Melipona quadrifasciata* obtained in São Paulo, and of *Schinus* (Anacardiaceae) in a sample of *Tetragonisca angustula* obtained in Minas Gerais. Accessory pollen belonged to the pollen types of *Myrcia* (Myrtaceae) and Melastomataceae/*Combretum* (Combretaceae).

Geopropolis from Ribeirão Preto, São Paulo State, was analyzed by Barth (2006). Six samples obtained from different bee species in the same locality showed a large diversity of pollen grains. Dominant pollen of *Eucalyptus* was detected in geopropolis of *Trigona recursa* alone. Geopropolis of *Lestrimellita limao* and *Nannotrigona testaceicornis* showed accessory pollen of *Eucalyptus*, *Melipona quadrifasciata* that of *Mimosa scabrella* pollen type and *Tetragonisca angustula* that of *Cecropia*.

Geopropolis of *Frieseomelita varia* showed no predominant pollen type. This fact indicated a foraging preference when visiting very heterogeneous vegetation in an area and vegetation reflecting strong human influence.

Archaeological geopropolis, obtained in the region of Januária, Minas Gerais State, indicated a period before *A. mellifera* was introduced into Brazil, and was studied by Barth et al. (2009). Vegetation types were inferred using the pollen spectra of geopropolis. The predominance of hygrophilous plants (*Chrysophyllum*, Sapotaceae; *Cedrela*, Meliaceae; *Cuphea*, Lythraceae; *Ludwigia*, Onagraceae and Myrtaceae) characterized the river gallery forests. A mixed vegetation of herbaceous plants, few trees and indicators of humid soils (*Cuphea thymoides*, Lythraceae) could indicate the presence of forest patches inside a field landscape. Pollen grains of several palm species in another geopropolis sample indicated a drier open landscape.

The great diversity of the foraging vegetation used by native bees was investigated in the South American countries of Bolivia, Brazil, and Venezuela by pollen analysis of geopropolis samples, mainly in the Amazon region (Freitas et al., 2012). Between the four Brazilian geopropolis analyzed, two were dominant for Melastomataceae (*Melipona*) and two had accessory pollen grains of Arecaceae and *Cecropia* (*Tetragonisca angustula*). Among four Bolivian, four Brazilian and eight Venezuelan geopropolis and propolis analyzed, dominant pollen of three plants was found in four samples: 1. Solanaceae (*Melipona grandis*, Bolivia). 2. Melastomataceae (*Melipona quadrifasciata* and *Melipona mondury*, Brazil). 3. *Melochia*, Sterculiaceae (*Tetragona clavipes*, Venezuela). Accessory pollen belonged to Arecaceae, *Cassia* (Caesalpiniaceae), *Cecropia* (Cecropiaceae), *Crotalaria* (Fabaceae), *Didymopanax* (Araliaceae), *Eucalyptus* (Myrtaceae), Fabaceae-Faboideae, *Inga* (Mimosaceae), *Myrcia* (Myrtaceae), Rubiaceae and *Tabebuia* (Bignoniaceae). A high number of pollen types (below 15% each one) was present in geopropolis and propolis.

Hyphae and fungal spores (Table 1) were nearly always present in geopropolis samples. They are possibly transported and deposited by the air on fresh and sticky resins before the bees collected them. Sand and clay fragments are collected to increase the strength of the primitive cerumen pots. Sandy fragments were found also in all samples, except in geopropolis samples from Ribeirão Preto, São Paulo State, produced by *Nannotrigona testaceicornis* and *Frieseomelita varia* bees (Barth, 2006). Propolis samples of these bees showed plant tissue fragments. They are residues of the interactions of bees with plants, as well as the amorph organic material. Plant tissue fragments were present in geopropolis samples from Bolivia, Brazil and Venezuela, except some ones collected by *Scaptotrigona polysticta* (Bolivia), *Tetragona clavipes* and *Melipona favosa* (Venezuela). All samples from Ribeirão Preto contained amorphous brownish organic material, which was less frequent in samples from other countries. Resins are visible if they are not dissolved by reagents. Resin fragments were observed in samples of *Melipona grandis*, *Scaptotrigona depilis*, and *Scaptotrigona polysticta* from Bolivia, *Melipona mondury*, *Melipona quadrifasciata* and *Tetragonisca angustula* from Brazil and *Lestrimelitta limao*, *Melipona favosa*, *Scaptotrigona* sp. and *Tetragona clavipes* from Venezuela (Freitas et al., 2012).

9.4 Conclusions

Pollen analysis of geopropolis and propolis confirm high diversity vegetation around the meliponaries. Comparing the information provided by pollen analysis of honey, bee pollen and resinous products, only bee pollen is 100% actively collected by the bees; honey and resins may contain anemophilous pollens (e.g. Poaceae) landed in natural materials before their transformation into honey, cerumen, geopropolis and propolis. Pollen grain identification in geopropolis samples is useful for recognition of the environment. Pollen analysis of geopropolis samples also aims to provide some information about the plant resources used by bees around the collection sites. Pollen studies and associated physicochemical analyses are important tools for a better characterization and quality certification of geopropolis (containing sand/clay fragments), propolis, and cerumen.

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Table 1. Evaluation of geopropolis samples for sediment constituents, excluding pollen grains

(+) present, (-) not detected, (0) no data. (F) *Frieseomelita* (L) *Lestrimelitta*, (M) *Melipona*, (N) *Nannotrigona*, (Te) *Tetragona*, (Tr) *Trigona*, (Tt) *Tetragonisca*, (S) *Scaptotrigona*.

Authors	Bee species	Hyphae and fungal spores	Sandy/clay fragments	Plant tissue fragments	Amorph organic material	Resin
Barth and Luz (2003)	*M. quadrifasciata*	+	+	-	+	-
	Tr. sp.	+	+	-	+	-
	M. quadrifasciata	+	+	-	+	-
	Tt. angustula	+	+	-	-	-
	M. favosa orbygnii	+	+	-	-	-
	Tt. angustula	+	+	-	+	+
	Tt. angustula	+	+	-	+	-
	Tt. angustula	+	+	-	+	-
	M. quadrifasciata	-	+	-	-	-
Barth (2006)	*L. aff. limao*	+	+	-	+	0
	Trigona recursa	+	+	-	+	0
	Tt. angustula	+	+	-	+	0
	M. quadrifasciata	+	+	-	+	0
	N. testaceicornis	+	-	+	+	0
	F. varia	+	-	+	+	0
Freitas et al. (2012)	*Tt. angustula*	+	+	+	+	+
	Tt. angustula	+	+	+	+	+
	M. quadrifasciata	+	+	+	+	+
	M. mondury	+	+	+	+	-
	S. depilis	+	+	+	-	-
	S. depilis	+	+	+	-	-
	S. polystica	+	+	-	-	+
	M. grandis	+	+	+	+	-
	Te. clavipes	+	+	+	-	+
	Te. clavipes	-	+	-	-	-
	Te. clavipes	+	+	+	-	-
	S. sp.	+	+	+	-	+
	L. limao	+	+	+	-	+
	M. favosa	+	+	+	-	+
	M. favosa	-	+	-	+	-
	M. favosa	-	+	-	+	+

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