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Artigo

Occurrence of filamentous fungi associated with stingless bees *Melipona* in meliponaries at the metropolitan region of Manaus, Amazonas

Ocorrência de fungos filamentosos associados a abelhas sem ferrão *Melipona* em meliponários da região metropolitana de Manaus, Amazonas

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Abstract. Microbiota is the set of microorganisms, mainly fungi and bacteria, that are usually associated with tissues and organs of animals or plants. Bee-associated microorganisms can interact with their host in different ways, but the hole of ectomicroorganisms still scarce. In order to evaluate the occurrence of filamentous fungi on the body surface of stingless bees (Melipona spp.), in artificial beehives of the metropolitan region of Manaus, bees of two meliponaries were studied. All screened bees showed the occurrence of fungi on their body surface, where the genera *Penicillium, Fusarium, Acremonium and Cladosporium* were more frequent, representing more than 70% of the colonies identified. It remains to be seen whether the fungal microbiota associated with stingless bees is transient, permanent or whether they are performing some specific function.
Keywords. *microorganism, filamentous fungi, Melipona, stingless bees.*

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Introduction

It is estimated that there can be more than 4000 genera of bees and around 25 to 30000 species distributed in different regions of the world, and Brazil is home of about 25% of these species (Michener, 2000). The stingless bees have around 600 species worldwide. Of the 56 bees valid genera, 33 are exclusively Neotropical, with 417 described species. In Brazil, there are 244 described species. Of this total, 114 species occur in the Amazon biome (Camargo and Pedro, 2013). The genus *Melipona* occur exclusively from southern South America to the mountains of northern Mexico (Souza et al., 2009a), with about 74 species described (Camargo and Pedro, 2013). However, despite many *Melipona* species can produce honey in sufficient quantity to be commercially exploited, few are created for this purpose (Silveira et al., 2002). The honey of *Melipona* species presents high medicinal value but have more water than the honey produced by the genus *Apis* Linnaeus, 1758 (bees with sting) and is propitious to fermentation, thus, must be consumed quickly and make its commercialization more complicated (Souza et al., 2004).

The development of a systematic colony breeding process of these bees' species may allow the economic exploitation of their products, which provides an alternative source of income for many local people (Souza et al., 2009a). Brazilian ecosystems, especially the Amazon, have

many conditions that favor the creation of stingless bees. Among them, we can mention: warm climate, diverse flora, which supplies nectar, pollen and resin, flowering distributed throughout the year, different species of honey bees and a large market with a good price for this product (Venturieri, 2008). Although the honey production of stingless bees is lower than that of *Apis mellifera* bee, meliponines have very important advantages over other species, especially because they are much more adapted to the pollination of trees in our forests (Venturieri, 2008).

Pollinators provide valuable services and resources for ecosystems, diseases associated with their disappearance pose a risk to human well-being, both directly and indirectly, by affecting livestock, agriculture and wildlife (Furst et al., 2014). Despite the importance of bees and other pollinators for the maintenance of fauna and flora structure, being responsible for 87.5% of pollination of the Amazonian native flora Ollerton et al. (2011), the abundance of stingless bees' species has decreased due to a negative influence of man on the environment. The fragmentation of natural environment leads to a decrease of shelter supply and foraging sites (decreasing honey production), destruction and/or predation of colonies and a sharp reduction in support capacity of forest reserve areas (Souza et al., 2009a). Other factors that may be influencing the disappearance of stingless bees are competition with African bees, the excessive use of chemicals in agriculture, the predation by other arthropods, and diseases associated with microorganisms such as viruses and fungi as well. All known insect species harbor a rich and complex community of microorganisms such as mites, protozoa, viruses, bacteria and fungi, where they can be found on their body surface or in their interior. However, our knowledge about the native stingless bees' reproduction and associated microbiota still scarce. One of the important aspects about the biology of these insects is the knowledge of the fungal microbiota, which can cause disease to these organisms (Ferraz et al., 2008). Although studies have reported on the microbiota of hives (Santos, 2007; Morais et al., 2013) and honey made by stingless bees in Brazil (Souza et al., 2009b; Matos et al., 2011), few studies have directly examined (Ferraz et al., 2008; Silva et al., 2011) and there are few reports on the relationship of fungi with stingless bees (Ferraz et al., 2008). In this context, a survey was carried out in order to know the diversity and incidence of filamentous fungi on the body surface of stingless bees in Central Amazon.

Material and Methods

The bees were collected in the season of highest rainfall, in March and during the dry season, in September of 2013, in two meliponaries (bees apiaries), one in the municipality of Manaus (Meliponário Sucupira 3º04'17.15 "S 59º53'10.27" W) and another in the municipality of Iranduba (Sítio dos Tucanos 3º13'02.00 "S 60º13'29.24" W), Amazonas, Brazil. Twenty-four live bees (*Melipona seminigra* end *Melipona interrupta*) were collected per season at each point using forceps. After individual collection, the individual was transferred to individual sterilized tubes, and packed in a refrigerated thermal box for material preservation. Overall 96 stingless bees were investigated.

Isolation of fungi was performed according to the methodology of King et al. (1979), with modifications. The dead bees were distributed in tubes containing 10 mL of 0.9% NaCl saline solution, vortexed and then 100 µL of the solution was transferred to Petri dishes containing Sabouraud Agar and Potato Agar Dextrose (BDA) with antibiotic solution amoxicillin 100 mg/L, pH 6.8, in triplicate and incubated in BOD at 28 °C for up to 30 days.

The fungi colonies were separated and purified by removing a small fragment of inoculum and transferring to a tube containing 1.0 mL of Tween 80 solution, vortexing until the solution homogenized (São José et al., 1994). Dilution was done serially, transferring 100 µL of the sample suspension into a tube containing 900 µL of sterile saline concentration (dilution 10-1), the dilution was then homogenized and repeated operation in successive dilutions until dilution of 10-3. At the end of the dilution series, 100 µL of the 10-3 dilutions were inoculated in triplicate and incubated at 28 °C for two days. The developing colonies were transferred to tubes, identified and incubated for eight days. The pure samples were collected in Petri dishes for further identification.

The microculture was performed on a slide, according to Riddell (1950) protocol, with modification. Filter paper was used on the bottom of Petri dish, on the paper was placed a pair of coverslips and a 76 x 26 mm sheet sterilized. Two blocks of BDA and or Malt Agar were transferred to the central surface of the slide, at the four ends of the agar block a portion of the colony was inoculated. The two sterile coverslips were deposited on the surface of the agar blocks. Sterilized distilled water was deposited on the bottom of the plate enough for five to seven days.

After fungal growth, the coverslips were carefully removed and placed on a drop of lactophenol-blue dye on the surface of a 76 x 26 mm slide and analyzed by optical microscopy (Onions et al., 1981) for identification of their Sexual and/or asexual structures (Ellis, 1971; Barnett and Hunter, 1972; von Arx, 1974).

We also calculate several assemblage's metrics that provide a more accurate description of the fungal diversity at each meliponarie in both seasons. To compare the dominance of fungi between the different sampling seasons, the data acquired from each site are presented as a percentage of abundance, modified by Ludwig and Reynolds (1988). At where:

i = 1, 2, 3,..., S
Pi = percentage of abundance of i genera
ni = number of colonies of fungi with i genera
S = number of genera found in all samples

$$Pi = \frac{ni}{\sum_{i=1}^{S} ni} \; x\; 100$$

To calculate the total *Pi* of the bees, *ni* corresponds to the number of fungal colonies of each genus present in the meliponaries in each season. The genera with percentage of abundance (*Pi*) greater than 5% were considered abundant and the others rare (*Pi* <5%).

The Shannon Index (*H'*) is a commonly used index that takes into account both abundance and evenness of organisms present in the community.

$$H' = -\sum_{i=1}^{S} (P_i)(In\ P_i)\Bigg)$$

For greater reliability, we used the Simpson Index (*D*), which measures the likelihood that two individuals randomly selected from one sample belong to the same species. It is an index often used to quantify the biodiversity of a habitat.

$$D = \sum_{i=1}^{S} P_i$$

The Pielou index (*J*) measures the equitability and allows the comparison of the Shannon Weaver index with the distribution of individuals in the observed species that would have maximum diversity.

$$J = \frac{H'}{In(S)}$$

We individually tested for differences in number of genera and fungal abundance between localities (Manaus and Iranduba meliponaries) using student T- tests. We also tested for differences fungal abundance between seasons using student T- tests. These analyses, assumes equivalent variances among samples. All data analyses were done in Excel, for windows.

Results and discussion

We isolated fungi in 100% the bees analyzed. Of the 297 colonies identified morphologically at the genus level, *Penicillium* (71), *Fusarium* (69), *Acremonium* (40) and *Cladosporium* (33) were the most predominant, surpassing 70% of those identified. Among the less frequent ones, *Torula* (2) and *Alysidium* (1) had a lower occurrence, being isolated only in the rainy season in Meliponário de Manaus (Table 1).

Both abundance (p = 0.956) and number of genera recorded (p = 0.455) were similar between Manaus and Iranduba meliponaries. A similar result was found polling the data from both localities. The abundance of fungal colonies was similar between seasons (p = 0.682).

The results obtained in the rainy season and dry season did not show significant variation by the Shannon indices (*H'* = 2.15 and 2.09), Simpson (*D* = 0.16 and 0.17) and Equitability (*J* = 0.04 and 0.04) (Table 3), since they were calculated having the genus as a reference taxonomic unit.

	MANAUS		IRANDUBA		Total
	RS	DS	RS	DS	
Penicillium	16.2	37.5	22.5	19.4	71
Fusarium	31.1	11.1	25.8	24.2	69
Acremonium	10.8	2.8	23.6	14.5	40
Cladosporium	2.7	20.8	4.5	19.4	33
Paecilomyces	9.5	6.9	5.6	4.8	20
Verticilium	1.4	4.2	10.1	1.6	14
Moniliella	4.1	2.8	1.1	4.8	9
Aspergillus	4.1	4.2	1.1	1.6	8
Rhizopus	1.4	1.4	3.4	3.2	7
Rhinocladiella	5.4	1.4	0	0	5
Tritirachhium	4.1	2.8	0	0	5
Scedosporium	2.7	1.4	1.1	1.6	5
Mucor	1.4	1.4	1.1	1.6	4
Pestalotiopsis	1.4	1.4	0	3.2	4
Torula	2.7	0	0	0	2
Alysidium	1.4	0	0	0	1
Total nº	74	72	89	62	297
Total genus	16	14	11	12	

RS = RAINY SEASON
DS = DRY SEASON

Table 1. Abundance (%) of filamentous fungi isolated from stingless bees (*Melipona* spp.) in the rainy and dry seasons of 2013 in the Meliponaries of Manaus and Iranduba, Amazonas-BR

The bees sampled during the rainy season represented 54.9% of the isolates, distributed in 16 genera. While the bees surveyed during the dry season represented 45.1% and 14 genera (Table 2). The highest abundance was observed in the rainy season, with emphasis on the genera *Fusarium* (28.2%), *Penicillium* (19.6%) and *Acremonium* (17.8%). In the dry season the genus *Penicillium* (29.1%) was also the most abundant, followed by *Cladosporium* (20.1%) and Fusarium (17.2%). The genus *Cladosporium* showed high abundance in the dry season, with a significant increase of 350% in comparison to the rainy season, in another slope, *Fusarium* showed a fall of 50%. Of the rare isolates in the rainy season, *Torula* (1.2%) and *Alysidium* (0.6%) were absent in the dry season (Table 2).

Despite, there was an overall higher number of fungal colonies sampled during the rainy season, decreasing in the following season, the variation between seasons were not significant. Moreira and Siqueira (2002) verified changes in fungal populations according to moisture levels, demonstrating that the higher the humidity, the greater the fungal populations. According to Ishikawa et al. (2012) in the rainy season - it is common to observe great diversity of fungi in the Amazon forest and Braga-Neto et al. (2008) pointed out that in the rainforest in the Ducke Reserve the higher the precipitation, the more fungi are recorded. This high number of fungi in bees in the seasons may be related to the season of greater foraging activity of stingless bees. The bee's external activities are concentrated in the morning, both in the rainy season from March

	MANAUS/IRANDUBA	
	RS	DS
Penicillium	19.6	29.1
Fusarium	28.2	17.2
Acremonium	17.8	8.2
Cladosporium	3.7	20.1
Paecilomyces	7.4	6.0
Verticilium	6.1	3.0
Moniliella	2.5	3.7
Aspergillus	2.5	3.0
Rhizopus	2.5	2.2
Rhinocladiella	2.5	0.7
Tritirachhium	1.8	1.5
Scedosporium	1.8	1.5
Mucor	1.2	1.5
Pestalotiopsis	0.6	2.2
Torula	1.2	0
Alysidium	0.6	0
Total nº	163	134
Tota occurrence %	54.9	45.1
Total genus	16	14

RS = RAINY SEASON
DS = DRY SEASON

Table 2. Occurrence (%) of filamentous fungi isolated from stingless bees (*Melipona* spp.) in the rainy seasons and dry seasons of 2013 in Meliponaries de Manaus and Iranduba, Amazonas-BR

to June, and in the dry season from September to December (Oliveira et al., 2012).

The high frequency of fungi in the analyzed bees confirms the diversity of these microorganisms on the body surface of these insects. Leão et al. (2012) studying stingless bees have reported that these insects are inhabited by a large diversity of microorganisms, but most of them are still unknown; Lima et al. (2012) and Morais et al. (2013) also highlighted the diversity of fungi associated with stingless bees.

In studies by Ferraz et al. (2008) and Lima et al. (2012), *Penicillium* and *Aspergillus* genus presented a high occurrence, however, in this study the genus *Penicillium* also had a high occurrence, but *Aspergillus* showed less frequency, with the same occurrence in both collection seasons. The genera *Fusarium*, *Acremonium* and *Verticillium* were not isolated by Ferraz et al. (2008) and Lima et al. (2012). Perhaps some factors may have influenced this occurrence: climate, habitat, different regions; or even the few reports of fungi associated with stingless bees.

The genera *Trichoderma, Monilia* and *Geotrichum* isolated by Lima et al. (2012); *Curvularia, Monilia, Nigrospora* and *Trichoderma*, by Ferraz et al. (2008), were not isolated in this study. Even showing low frequency Moniliella, Rhinocladiella, Tritirachium, Scedosporium, Pestalotiopsis, Torula and Alysidium had not yet been reported in stingless bees before. Rhizopus and Mucor were rarely isolated from stingless bees. Only Eltz and Gorke (2002) reported Rhizopus on stingless bees.

In many cases, the interaction between stingless bees and fungal microbiota may be viewed as mutualists (Morais et al. 2013), while in others can be classified as commensals. Menezes et al. (2015) report the first record of symbiosis between a stingless bee and cultivated fungus, and Oliveira et al. (1996 and 1999) describe that resins used by solitary or social bees to construct and protect their nests has inhibitory activity against some microorganisms such as bacteria and fungi. Marsaioli et al. (1998) isolated symbiotic filamentous fungi on the body surface of stingless bees, these fungi showed resistance to the nest's antimicrobial activity, but these fungal isolates presented promising antimicrobial activity.

From the fungus perspective, the bees may be facilitating the spore dispersion. Bees can travel a distance of 3 km and visit dozens of flowers from several plant species in search of food (Almeida et al., 2003). Therefore, the dispersion of the high number of fungi isolated from the body surface of these insects in these studies may be favored. The high cost of the molecular tools and the difficulty in morphological identification at the species level, hinders studies with fungi. The importance of knowing the fungal microbiota of stingless bees is fundamental to relate the interaction not yet understood between these organisms.

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		Genus	Simpson (D)	Equitability (J)	Diversity Shannon (H')
MANAUS/IRANDUBA	RS	16	0.16	0.04	2.15
	DS	14	0.17	0.04	2.08

RS = RAINY SEASON
DS = DRY SEASON

Table 3. Diversity of filamentous fungi isolated from stingless bees (*Melipona* spp.) in the rainy seasons and dry seasons of 2013 in Meliponaries de Manaus and Iranduba, Amazonas-BR

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