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An Alien in the Group: Eusocial Male Bees Sharing Nonspecific Reproductive Aggregations

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ABSTRACT. Sexual selection predicts that individuals competing for access to sexual partners should maximize their chances of mating by looking for sites where the chances of finding partners are more likely to occur. However, males of stingless bees have been observed sharing nonspecific reproductive aggregations. This uncommon behavior appears to confer no obvious increase of individual fitness. It has been suggested that this reproductive strategy is due to the similarity between male odors common to different stingless bee species. Cuticular hydrocarbons (CHCs) are candidate odors of interest because their nonvolatile pheromone nature allows them to play an important role in sexual behavior and species recognition. Here, we review the literature to evaluate whether any phylogenetic patterns exist among male stingless bees that aggregate with closely or distantly related species. We also compared the CHC profiles of males of Neotropical stingless bee species (*Plebeia* sp. Schwarz, *Trigona spinipes* (F.), *Tetragona clavipes* (F.), *Nannotrigona testaceicornis* (Lepeletier), *Scaptotrigona* aff. *depilis* (Moure), *Tetragonisca angustula* (Latreille), and *Melipona subnitida* (Ducke) to reveal any chemical similarities among their male odors. We found males of 21 stingless bee species involved in interspecific interactions mainly from Neotropical and Indo-Malayan/Australasian regions. Alien males did not necessarily visit host aggregations of closely related species. Furthermore, the CHC profiles of different studied species were very distinct from each other and do not overlapped at all. It is unclear yet why this apparently nonadaptive behavior carried out by some stingless bee males.

Key Words: Apidae, Behavior, Chemical Ecology, Hymenoptera, Phylogeny

Many male insects aggregate near nests or other sites where sexually receptive virgin females can be found, and where mating usually occurs (Emlen and Oring 1977, Sullivan 1981, Thornhill and Alcock 1983, Sivinski and Petersson 1997). One would expect that such reproductive aggregations would only contain individuals from the same species. In this way, sexual selection could maximize the individual fitness due to the abundance of members of the opposite sex of their species (Darwin 1859). However, while selective pressure on organisms should always optimize the search for sexual partners on species-specific aggregations to avoid any possibility of interspecific mating, this is not always the case. For example, although rare, mixed mating swarms in insects have been well documented in ants, bees, and wasps (Leprince and Francoeur 1986, Nadel 1987, O'Neill 1994, Koeniger and Koeniger 2000, Starr and Vélez 2009, Bänziger and Khamyotchai 2014, Santos et al. 2014).

Particularly in bees, this issue has been rather well illustrated. For example, it has been observed in mixed aggregations of males of three different *Centris* species (Centridini oil bees) predominantly compounded by *Centris decolorata* males, and also by males and females of *C. smithii* and *C. lanipes* (Starr and Vélez 2009). In honeybees (Apini) and stingless bees (Meliponini), some males (or drones) may visit aggregations of other bee species, as well. For example, *Apis cerana* drones shared *Apis dorsata* drone congregation area and vice versa (Koeniger and Koeniger 2000). Yet, in stingless bees, alien males of several species have been observed gather in nonspecific (host) reproductive aggregations (Schwarz 1948; Nogueira-Neto 1954; Kerr et al. 1962; Cortopassi-Laurino 1979, 2009; Bänziger and Khamyotchai 2014; Santos et al. 2014). These examples in bees show us an uncommon sexual behavior, since it is expected that males of only the same species would aggregate for reproductive purposes.

To understand a little more about possible causes for these uncommon sexual behaviors assumed by bee males, we discuss it here using stingless bees as model due rather common occurrence in this bee group. The mating system in stingless bees is lek-like because males congregate on a communal display area for the sole purpose of mating (Emlen and Oring 1977). However, the mating system can diverge from Emlen and Oring's definition because sexually receptive females (virgin queens) of stingless bees do not directly choose a certain male according to his status position or resource defense within aggregation. Instead, males must be able to pursue and achieve virgin queens during nuptial flight before their competitors (Roubik 1990).

The male aggregation in stingless bees appears to be initiated by the presence of virgin queens within the nest, which can promote the arrival of dozens, or even hundreds of conspecific males. This progressively forms a large aggregation of individuals that wait close to the nest for the nuptial flight of virgin queens (Michener 1946, Kerr et al. 1962, Roubik 1990, Velthuis et al. 2005, Cortopassi-Laurino 2007). At the moment of copulation, male stingless bees are involved in a suicidal mating because their seminal vesicles and genitalia remain attached within the female reproductive tract (Kerr et al. 1962). It not only kills them but also effectively prevents subsequent copulations by other males. Such behavior then becomes obligatory monogamist males and stingless bee queens essentially monandrous, according to observational and molecular data (Kerr et al. 1962, Strassmann 2001, Jaffé et al. 2014).

Selective pressure over stingless male bees can be still stronger. These individuals are involved in a competition among themselves to present better physical vigor, visual perception and chemical discrimination to detect virgin queens during their nuptial flights (Roubik 1990). It has been believed, then, to result in a strong selective pressure

on these physiological, anatomical, and behavioral traits (Roubik 1990). Therefore, visiting and staying in a reproductive aggregation compounded of other stingless bee species, as eventually can occur does not seem to be a good competitive strategy.

Some hypotheses could be raised to explain this uncommon male behavior in social bees. First, males of multiple bee species could be attracted by a common highly volatile chemical compound released by the queens. In honeybee species, studies demonstrated that nonsympatric and unspecific drones of *A. cerana*, *A. dorsata*, and *A. florea* can be attracted by the main sex queen pheromone of honeybees (*A. mellifera*), (E)-9-oxodec-2-enoic acid (Butler et al. 1967, Sannasi and Rajulu 1971, reviewed by Koeniger and Koeniger 2000). Second, males of multiple bee species could be attracted to a same local due to the odor released inside colonies. It was proposed by Velthuis et al. (2005) to explain why males of multiple stingless bee species often are seen aggregating themselves together. However, it is not clear which individuals could be potential releasers of such chemical compounds inside colonies. Third, males of different bee species share chemical profiles based on nonvolatile cuticular compounds. This hypothesis was suggested by Kerr et al. (1962) to explain why stingless bee males (alien species) could mistakenly join others males (host species) during reproductive aggregations.

Since male stingless bees may interact with each other in aggregations such as performing antennation or trophallaxis (Cortopassi-Laurino 2007, Santos et al. 2014, Supp Video 1 [online only]), it creates a scenario where they could recognize other species through profiles of cuticular hydrocarbons (CHCs). The CHCs are chemical compounds that have evolved primarily to provide a water-impermeable layer over an insect's epicuticle to protect them from desiccation and to act as a barrier against microorganisms (Wigglesworth 1957, Hadley 1994). Furthermore, these compounds have assumed multiple functions as signals of colony membership, age, sex, caste, and species, mainly in social species (Blomquist et al. 1998; Dani 2006; Nunes et al. 2008; Provost et al. 2008; Tannure-Nascimento et al. 2008; Ferreira-Caliman et al. 2010, 2013; Nascimento and Nascimento 2012). Therefore, CHCs can be considered ideal candidates to find any chemical similarity among males of multiple stingless bee species.

To date, no studies have been conducted to compare the male odors of different stingless bee species and to determine whether male CHC profiles are sufficiently similar among different species to explain why some males join nonspecific aggregations. That approach may offer opportunities to further our understanding of the evolution and maintenance of this lek-like mating system and offer parallels with the male aggregation behaviors observed in other insect orders (Sullivan 1981, Thornhill and Alcock 1983, Sivinski and Petersson 1997).

Here, we review the literature to find any phylogenetic pattern in the occurrence of male stingless bees visiting nonspecific aggregations. Second, we compared the CHC profiles of stingless bee males of different species. Our main goals were to organize this knowledge, i.e., whether males of different stingless bee species have been aggregated together and whether CHC profiles could give us any indication of a likely chemical similarity among species.

Materials and Methods

Interspecific Male Interactions. We reviewed the literature dealing with alien males participating in other host male reproductive aggregations of stingless bee species. To visualize these data, we used the Cytoscape software (Shannon et al. 2003). In Cytoscape, we used the Network Analyzer function. After that, we generated styles of networks to link the number of direct edges (i.e., whether there was an interaction between species' pairs and its direction). Then, data visualization was done for mapping node size ascribing low values to small sizes (few interactions) and high values to greater interactions between alien and host species. Subsequently, we applied workflow function in Cytoscape followed by the group attributes layout and, finally, wordcloud plug-in where all words (here, species' names) were calculated

proportionally according to their frequency in the input text (Saito et al. 2012). Thus, this approach allowed us to integrate and to connect possible relationships according to the data reported in the literature.

During visual data making and by considering its respective interaction (from alien to host species) according to literature, we organized the stingless bee species according to their distribution from Neotropical, Afrotropical, and Indo-Malayan/Australasian regions.

Chemical Profiles. Ten males of every *Plebeia* sp. Schwarz, *Trigona spinipes* (F.), *Tetragona clavipes* (F.), *Nannotrigona testaceicornis* (Lepeletier), *Scaptotrigona* aff. *depilis* (Moure), *Tetragonisca angustula* (Latreille), and *Melipona subnitida* Ducke stingless bee species were stored separately in glass vials and kept at 4°C prior to the chemical analysis. All of the species were collected during reproductive aggregations between August 2009 and April 2012, in São Paulo state, Brazil, except for *M. subnitida* that was collected in Rio Grande do Norte state, Brazil. Voucher specimens were deposited at Coleção Entomológica Paulo Nogueira-Neto from Bee Lab Universidade de São Paulo, in São Paulo/Brazil.

The cuticular compounds of each male bee were extracted in 1 ml hexane for 1 min. After evaporation of the solvent under a stream of N₂, the nonpolar extract was resuspended in 100 µl of hexane and 1 µl sample was injected into a combined gas chromatography-mass spectrometry system (CGMS, Shimadzu, model QP2010, Kyoto, Japan). The separation was carried out on a 30 m Rtx-5MS column with helium as the gas carrier at a flow rate of 1.0 ml min⁻¹. The oven temperature was initially set to 150°C, and then increased by 3°C min⁻¹ until it reached 280°C (hold time = 15 min). Analyses were performed in splitless mode. The mass spectra were obtained by 70 eV ionization.

Mass spectra were analyzed using GCMS Solutions for Windows version 2.6 (Shimadzu Corporation, Brazil). The chemical compounds were identified based on their mass spectra by comparison with Nist Library 08 data and with a standard solution of different synthetic hydrocarbons (Fluka).

Data Analysis: Male Aggregation Analysis. First, we did a binomial test to evaluate whether stingless bee males aggregate themselves with their own species or not. If so, we estimated the probability on whether there could be a significantly higher number of cases of males from closely related species (here, same genus) participating in a nonspecific aggregation than by chance. For both cases, we applied a binomial test by using the function "binom.test" from stats package for R (R Core Team 2014). In the first case, the success number was considered as the number of observations of aggregations receiving alien species versus the total amount of male aggregations observed in literature. In the second case, the success number was considered as the number of observations of males visiting aggregations of closely related species versus distantly related species. For both, we hypothesized a probability of success of 0.8, an alternative hypothesis as two-sided and a confidence interval of 0.95 (95% CI). Data were visualized using Euler diagram provided by venneuler package (Wilkinson 2011) for R.

Data Analysis: Chemical Analyses. The relative abundance of each compound was estimated from the proportion of the peak area of the total ion chromatograms. We compared the chemical composition of the cuticular surface lipids of males using nonparametric multivariate analyses using PAST version 3.04 (Paleontological Statistics Software Package, Hammer et al. 2001). We performed a nonmetric multidimensional scaling (NMDS) analysis based on the Bray-Curtis distances to generate a graphic showing the differences between species. Bray-Curtis distances were also used to plot a cluster analysis and to make a one-way analysis of similarities (ANOSIM) with 9,999 permutations to test for significance in dissimilarities between the chemical profiles of males of different species.

R-value is based on similarity matrices (or distances) ordering the values to perform the statistical procedure. *R*-values between 0 and 1 indicated the level of similarity, where *R* = 0 indicates no difference between the groups and *R* = 1 indicates a larger similarity within a group than between groups. *P* values were adjusted using the

Bonferroni correction. Similarity percentages (SIMPER) were calculated to identify the compounds that predominantly contributed to the Bray-Curtis dissimilarities among species.

Results

Alien Males in Host Aggregations. Our review found at least 65 reproductive aggregations of stingless bees (Supp Table 1 [online

only]) of which ~71% received only one single species, and 29% received males of different stingless bee species at any time (Fig. 1). There was a significant difference between those aggregations formed by only one species and with those having two or more species (Exact Binomial Test, CI: 0.18–0.41; $P < 0.0001$). In these later kind of aggregations (two or more species), just a few percentage of alien males of stingless bee species (circa 18%, Fig. 1) were phylogenetically closely related to the host species. Finally, our binomial test showed that the probability of males joining aggregations of another stingless bee species is 21%, and may occur by chance (Exact Binomial Test, CI: 0.06–0.45; $P < 0.0001$).

Overall, we found 14 Neotropical, one Afrotropical, and six Indo-Malayan/Australasian stingless bee species visiting in any time aggregations of another species (Fig. 2, Supp Table 1 [online only]). Males of the Neotropical, *Scaptotrigona* spp. and Indo-Malayan/Australasia, *Tetragonula* spp. and *Lepidotrigona* spp. showed the most records in the literature of individuals visiting nonspecific aggregations or receiving alien stingless bee species in their reproductive aggregations. Only *Scaptotrigona*, *Melipona*, and *Lepidotrigona* have been reported, to date, of receiving the visit of closely related genera males (references in Supp Table 1 [online only]). The oddest observation was when an Afrotropical male stingless bee, *Melipona bocandei* (Spinola), was recorded over five consecutive days in a male aggregation of the Neotropical bee *Scaptotrigona postica* (Latreille) (Fig. 2, Supp Table 1 [online only]).

During this study, we also observed two other uncommon behaviors. In September 2009, we made a video recording of a single male *M. scutellaris* visiting and peacefully interacting with a reproductive aggregation of *Scaptotrigona* aff. *depilis* males at the apiary of the Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, a part of the University of São Paulo, in Ribeirão Preto (Supp Video 1 [online only]). Again in July 2011, in the Universidade de São Paulo’s apiary, São Paulo, we recorded more than 30 *T. clavipes* males landing throughout the day on three consecutive days at one colony *S. aff. depilis* (Supp Fig. 3 [online only]). However, the *S. aff. depilis* males were

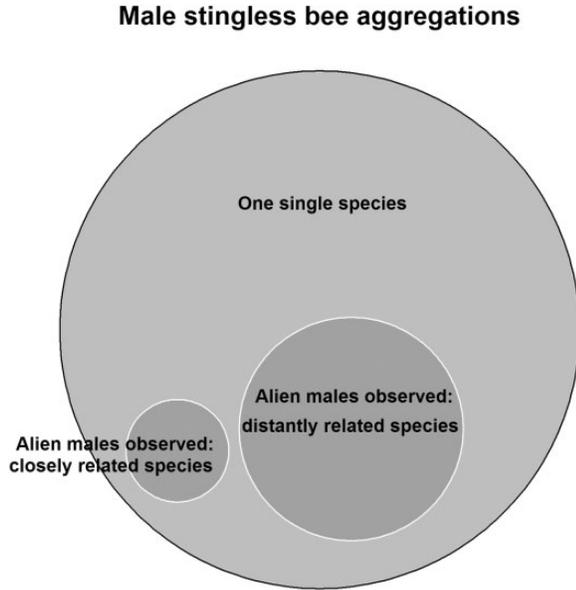


Fig. 1. Euler diagram showing male stingless bee aggregations compounded by one single species and relative proportion of those aggregations where males of other stingless bee species have been seen visiting closely or distantly related species.

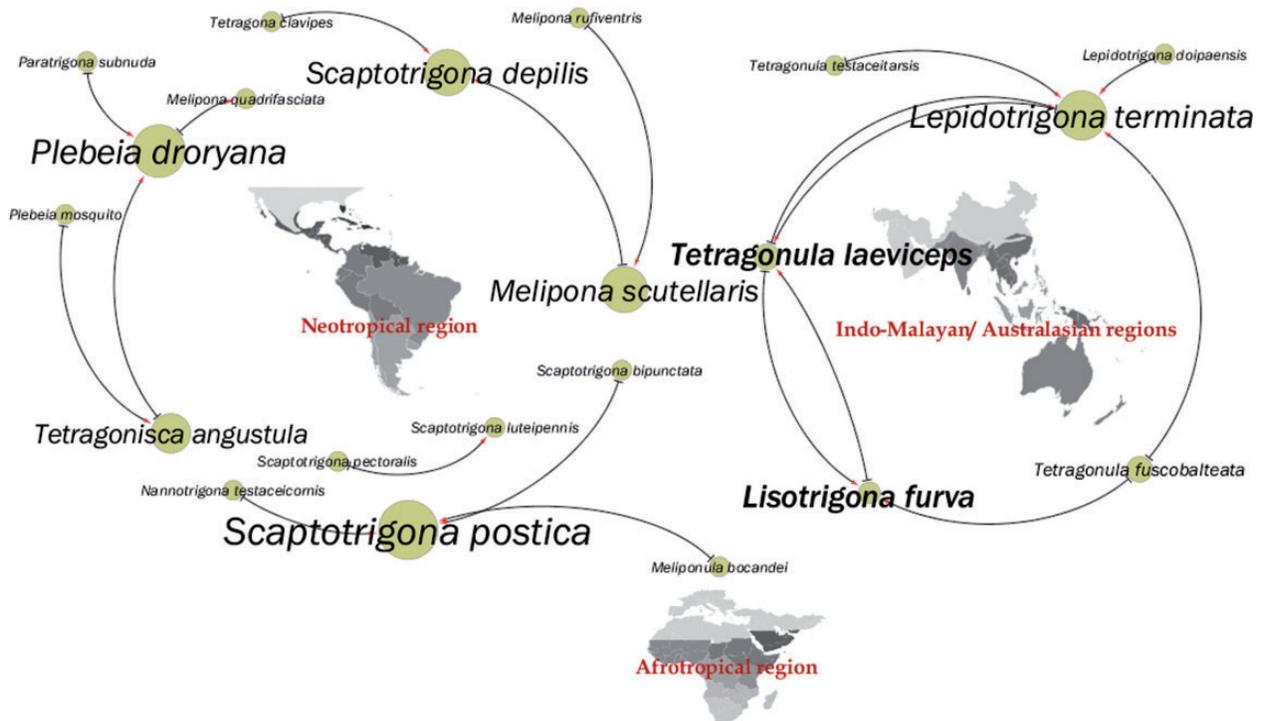


Fig. 2. Network interactions among males of different stingless bee species sharing mixed-species reproductive aggregations noted in the literature. Notice that although males may aggregate with closely related species, they may also visit more distantly related stingless bee species. Origin of lines (T) indicates those stingless bee species (alien) whose males visited nonspecific (host) aggregations (red arrows). The larger the circle and the letter most frequently species was observed receiving or visiting other stingless bee species.

Table 1. Relative concentrations (mean, SD) of CHCs in males of *Plebeia* sp., *T. clavipes*, *N. testaceicornis*, *M. subnitida*, *T. spinipes*, *T. angustula*, and *S. aff. depilis*

Hydrocarbons	RT (min)	<i>Plebeia pugnax</i>	<i>T. clavipes</i>	<i>N. testaceicornis</i>	<i>M. subnitida</i>	<i>T. spinipes</i>	<i>T. angustula</i>	<i>S. aff. depilis</i>
Heneicosane (<i>n</i> -C ₂₁)	18.054	0.11 ± 0.09	0.26 ± 0.35	—	0.09 ± 0.04	—	—	—
Docosane (<i>n</i> -C ₂₂)	19.944	—	—	—	—	—	0.09 ± 0.08	—
Tricosene*	22.765	—	—	—	0.22 ± 0.50	0.37 ± 0.33	—	—
Tricosane (<i>n</i> -C ₂₃)	23.546	3.93 ± 1.33	1.35 ± 0.66	3.42 ± 0.54	1.77 ± 0.92	1.87 ± 0.92	0.28 ± 0.20	0.82 ± 0.29
Tetracosene	25.444	—	0.21 ± 0.56	—	—	0.22 ± 0.25	—	—
Tetracosane (<i>n</i> -C ₂₄)	26.199	—	0.80 ± 0.28	0.11 ± 0.09	0.45 ± 0.11	0.50 ± 0.15	0.32 ± 0.12	0.46 ± 0.10
7-Methyl C ₂₄	26.897	0.74 ± 0.28	—	0.50 ± 0.13	0.38 ± 0.16	—	—	—
Pentacosene ^a	28.073	—	—	0.14 ± 0.17	1.14 ± 0.83	1.05 ± 0.30	—	0.53 ± 0.41
Pentacosene ^b	28.291	—	—	0.09 ± 0.10	0.06 ± 0.05	0.32 ± 0.11	—	—
Pentacosene ^c	28.419	0.54 ± 0.99	—	—	0.07 ± 0.08	—	—	—
Pentacosane (<i>n</i> -C ₂₅)	28.836	19.57 ± 6.32	50.07 ± 4.23	13.03 ± 1.83	25.94 ± 4.07	30.40 ± 6.16	46.78 ± 7.32	21.88 ± 3.04
13-; 11- Methyl C ₂₅	30.137	—	0.15 ± 0.13	1.37 ± 0.41	—	—	—	—
5-Methyl C ₂₅	30.500	—	—	0.15 ± 0.18	—	—	—	—
3- Methyl C ₂₅	31.299	—	—	0.53 ± 0.94	—	—	—	—
Hexacosene	30.610	—	0.13 ± 0.07	—	0.11 ± 0.03	—	—	—
Hexacosane (<i>n</i> -C ₂₆)	31.300	—	0.74 ± 0.12	0.45 ± 0.12	0.78 ± 0.09	1.33 ± 0.13	1.45 ± 0.14	0.90 ± 0.17
Heptacosene ^a	33.090	1.29 ± 0.69	—	3.83 ± 7.78	2.71 ± 0.70	2.60 ± 0.65	0.55 ± 0.63	24.00 ± 3.48
Heptacosene ^b	33.282	—	0.92 ± 0.23	0.22 ± 0.18	0.18 ± 0.09	0.30 ± 0.10	—	0.96 ± 0.16
Heptacosene ^c	33.745	—	—	—	0.11 ± 0.03	—	—	—
Heptacosane (<i>n</i> -C ₂₇)	33.773	10.21 ± 2.46	18.81 ± 1.38	19.89 ± 2.38	18.22 ± 1.95	46.18 ± 3.50	31.66 ± 2.92	14.37 ± 2.53
13-Methyl C ₂₇	34.850	—	—	2.49 ± 0.74	—	0.14 ± 0.07	—	0.73 ± 0.35
9-Methyl C ₂₇	35.020	—	—	0.12 ± 0.12	—	—	0.09 ± 0.21	—
7-Methyl C ₂₇	35.448	—	—	0.18 ± 0.14	—	—	—	—
Octacosene	35.474	—	0.50 ± 0.65	1.56 ± 0.21	0.88 ± 0.12	—	—	—
Octacosane (<i>n</i> -C ₂₈)	36.089	—	0.70 ± 0.10	0.18 ± 0.14	0.45 ± 0.09	0.53 ± 0.11	0.68 ± 0.15	0.68 ± 0.13
Nonacosadiene ^a	37.134	—	—	0.13 ± 0.12	1.35 ± 0.60	0.20 ± 0.08	—	—
Nonacosadiene ^b	37.300	—	—	—	0.23 ± 0.10	—	—	—
Nonacosene ^a	37.605	18.78 ± 7.81	—	—	0.29 ± 0.11	—	—	—
Nonacosene ^b	37.859	6.60 ± 3.97	0.10 ± 0.11	37.34 ± 5.81	25.87 ± 3.89	0.63 ± 0.16	0.75 ± 1.59	15.29 ± 6.12
Nonacosene ^c	37.995	—	0.13 ± 0.06	1.29 ± 0.25	0.57 ± 0.53	0.15 ± 0.09	—	—
Nonacosane (<i>n</i> -C ₂₉)	38.378	5.83 ± 1.81	15.88 ± 2.85	3.31 ± 0.48	6.93 ± 1.68	8.44 ± 2.40	9.15 ± 1.98	8.97 ± 3.39
13-; 11-Methyl C ₂₉	39.030	0.29 ± 0.51	3.41 ± 2.89	0.94 ± 0.30	0.19 ± 0.08	0.10 ± 0.07	—	0.69 ± 0.97
Triacotene*	40.085	—	—	0.10 ± 0.11	0.41 ± 0.08	0.46 ± 0.44	—	—
Triacotane (<i>n</i> -C ₃₀)	40.585	—	0.39 ± 0.12	0.15 ± 0.25	0.07 ± 0.04	0.95 ± 0.68	0.51 ± 0.17	0.27 ± 0.13
?-Methyl C ₃₀	40.620	—	—	—	—	—	0.09 ± 0.14	—
Hentriacontadiene ^a	41.506	0.91 ± 0.49	—	0.27 ± 0.26	1.22 ± 0.43	0.53 ± 0.26	—	—
Hentriacontadiene ^b	41.613	0.47 ± 0.30	—	3.28 ± 1.08	0.54 ± 0.22	0.26 ± 0.21	—	—
Hentriacontene ^a	41.956	16.15 ± 3.41	—	0.69 ± 0.40	0.29 ± 0.11	0.19 ± 0.22	—	5.24 ± 1.81
Hentriacontene ^b	42.141	5.98 ± 1.27	—	2.26 ± 0.50	—	0.98 ± 0.54	—	—
Hentriacontene ^c	42.227	6.70 ± 2.55	0.30 ± 0.72	0.85 ± 0.60	7.67 ± 1.59	0.10 ± 0.07	—	—
Hentriacontane (<i>n</i> -C ₃₁)	42.716	1.03 ± 0.43	5.16 ± 1.87	0.47 ± 0.07	0.85 ± 0.53	0.78 ± 0.70	6.44 ± 2.54	2.87 ± 5.23
?-Methyl C ₃₁	42.022	—	—	0.26 ± 0.21	—	0.30 ± 0.16	0.62 ± 0.65	—
Dotriacontane (<i>n</i> -C ₃₂)	43.449	—	—	—	—	—	0.26 ± 0.17	1.05 ± 2.87
Trtriacontene*	46.589	0.90 ± 0.52	—	0.39 ± 0.30	—	0.09 ± 0.06	—	—
Trtriacontane (<i>n</i> -C ₃₃)	44.640	—	—	—	—	—	0.12 ± 0.12	0.27 ± 0.28

*Double bonds not identified.
RT, retention time (min).

absent therein but they could be seen aggregating in front of a colony of their species 20 m away.

Analysis of CHCs Profiles. Analyses of male bee CHCs showed a total of 45 hydrocarbons, which varied in combinations among species. They were classified as linear alkanes, alkenes, alkadienes and methyl-branched alkanes and ranged between 21 and 33 carbon atoms (Table 1). Some hydrocarbons were present in only one or two bee groups. The most abundant compounds that were common to all groups were pentacosane, heptacosane, and nonacosane. The species with the least number of compounds was *T. angustula* (17 hydrocarbons). Males of *Plebeia* sp. and *S. aff. depilis* had 18 CHC compounds, while males of *T. clavipes* had 19 compounds. *N. testaceicornis*, *M. subnitida*, and *T. spinipes* showed the highest number of CHCs: 28, 31, and 33, respectively.

The Bray-Curtis similarity cluster analysis shows that all individuals are correctly allocated to their predicted group, except for a single male of *N. testaceicornis* (Fig. 3). According to the cluster analysis, males of *T. clavipes* were more similar to those of *T. angustula*, and males of *N. testaceicornis* were more similar to those of *M. subnitida*. *T. spinipes* males were similar to *T. clavipes* and *T. angustula* males. Finally, *S. aff. depilis* males were similar to *N. testaceicornis*, and *M. subnitida*. *Plebeia* sp. was the group that showed the highest distance among the other species (Fig. 3).

NMDS demonstrated that males showed species-specific differences in their chemical profiles (Fig. 4). The NMDS plot showed a lower dissimilarity among males of *T. clavipes*, *T. angustula*, and *T. spinipes* and males of *N. testaceicornis*, *M. subnitida*, and *S. aff. depilis*. In contrast, males of *Plebeia* sp. were well separated from the other groups of males in the NMDS plots, thus supporting the Bray-Curtis similarity measure.

ANOSIM showed that the CHC profiles of males exhibited large dissimilarities, with global $R = 0.9632$, $P < 0.0001$ (Table 2). The values for the Bray-Curtis dissimilarity indices in the CHC profiles of pairs of males were >0.90 , except for *T. clavipes* versus *T. angustula* ($R = 0.8769$). R and P values in ANOSIM and the compounds that predominantly contribute to the Bray-Curtis dissimilarities are shown in Table 2.

Discussion

Our literature review showed that, with few exceptions, alien male stingless bees visiting host species aggregations do not necessarily associate with closely related species (Schwarz 1948; Nogueira-Neto 1954; Kerr et al. 1962; Cortopassi-Laurino 1979, 2009; Bänziger and Khamyotchai 2014; Santos et al. 2014). It is known that phylogenetically more related stingless bee species tend to share similar CHCs profiles among themselves (Leonhardt et al. 2013). However, our analysis

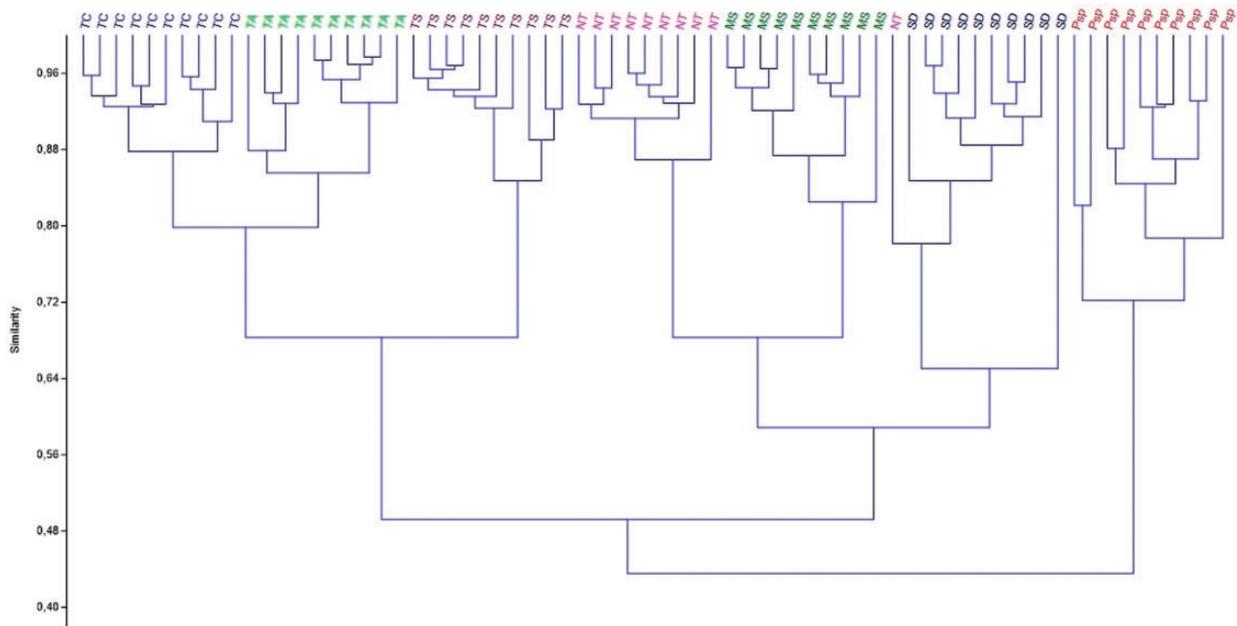


Fig. 3. Cluster analysis using the Bray-Curtis similarity measure. TC, *Tetragona clavipes*; TA, *Tetragonisca angustula*; TS, *Trigona spinipes*; NT, *Nanotrigona testaceicornis*; MS, *Melipona subnitida*; SD, *Scaptotrigona aff. depilis*; PP, *Plebeia sp.*

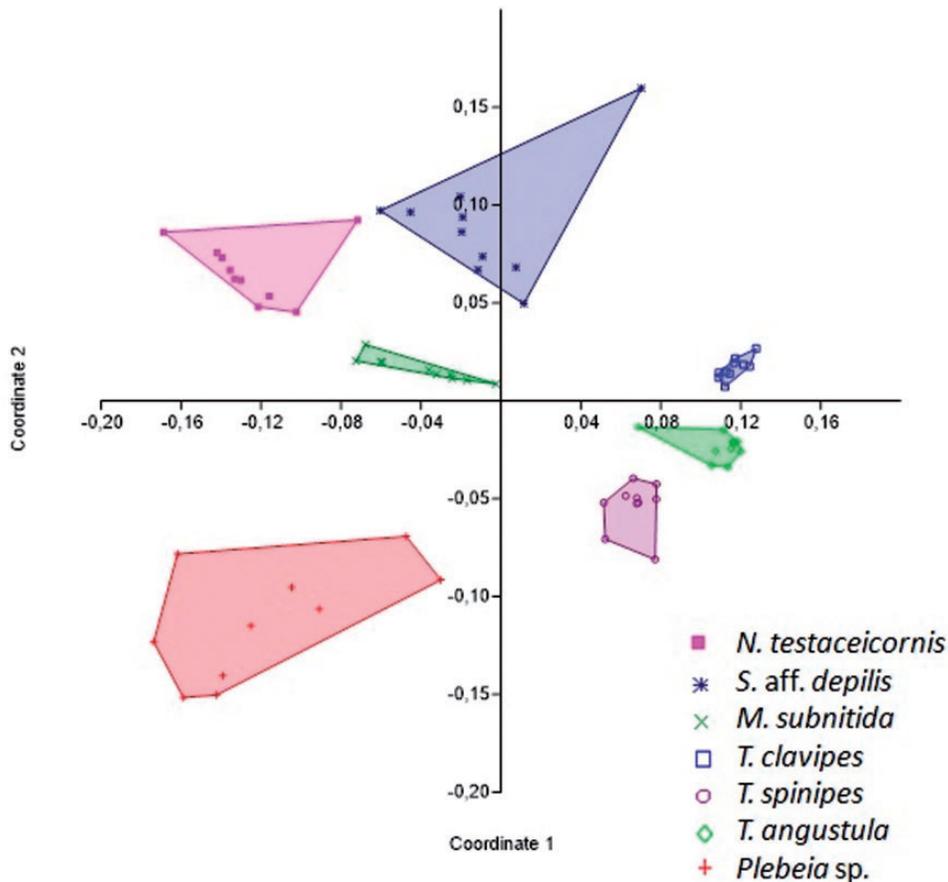


Fig. 4. NMDS results showing the differences in the chemical profiles of CHCs of male stingless bees.

showed that despite it some stingless male bees may randomly aggregate together to distantly related species.

Our chemical analyses (NMDS and cluster analysis) provided evidence for a clear distinction in CHC profiles among the different stingless bee species analyzed. Thus, there is not any chemical overlap that

could confuse males to aggregate with individuals of different stingless bee genera/species as suggested by Kerr et al. (1962). Nevertheless, there were some particular compounds (e.g., tricosane, pentacosane, heptacosane, and hentriacontane) which were common to the males of all the stingless bee species analyzed. However, such CHCs are rather

Table 2. Results of ANOSIM and SIMPER analysis of the cuticular compounds of Meliponini males

	R-values	P-values	Compounds that predominantly contribute to Bray-Curtis dissimilarities
<i>P. pugnax</i> vs <i>T. clavipes</i>	1.0	< 0.0021	<i>n</i> -C ₂₅ , Nonacosene ^{*a} , Hentriacontene ^{*a}
<i>P. pugnax</i> vs <i>N. testaceicornis</i>	1.0	< 0.0021	Nonacosene ^{*b} , Nonacosene ^{*a} , Hentriacontene ^{*a}
<i>P. pugnax</i> vs <i>M. subnitida</i>	1.0	< 0.0021	Nonacosene ^{*b} , Nonacosene ^{*a} , Hentriacontene ^{*a}
<i>P. pugnax</i> vs <i>T. spinipes</i>	1.0	< 0.0021	Nonacosene ^{*a} , Hentriacontene ^{*a} , <i>n</i> -C ₂₅
<i>P. pugnax</i> vs <i>T. angustula</i>	1.0	< 0.0021	<i>n</i> -C ₂₅ , <i>n</i> -C ₂₇ , Nonacosene ^{*a} , Hentriacontene ^{*c}
<i>P. pugnax</i> vs <i>S. depilis</i>	0.9969	< 0.0021	Heptacosene ^{*a} , Nonacosene ^{*a} , Hentriacontene ^{*a}
<i>T. clavipes</i> vs <i>N. testaceicornis</i>	1.0	< 0.0021	Nonacosene ^{*b} , <i>n</i> -C ₂₅ , <i>n</i> -C ₂₉
<i>T. clavipes</i> vs <i>M. subnitida</i>	1.0	< 0.0021	Nonacosene ^{*b} , <i>n</i> -C ₂₅ , <i>n</i> -C ₂₉
<i>T. clavipes</i> vs <i>T. spinipes</i>	1.0	< 0.0021	<i>n</i> -C ₂₇ , <i>n</i> -C ₂₅
<i>T. clavipes</i> vs <i>T. angustula</i>	0.8769	< 0.0021	<i>n</i> -C ₂₇ , <i>n</i> -C ₂₅ , <i>n</i> -C ₂₉
<i>T. clavipes</i> vs <i>S. depilis</i>	0.9973	< 0.0021	<i>n</i> -C ₂₅ , Heptacosene ^{*a} , Nonacosene ^{*b}
<i>N. testaceicornis</i> vs <i>S. depilis</i>	0.9609	< 0.0021	Nonacosene ^{*b} , Heptacosene ^{*a}
<i>N. testaceicornis</i> vs <i>M. subnitida</i>	0.9078	< 0.0021	<i>n</i> -C ₂₅ , Nonacosene ^{*b} , Hentriacontene ^{*c}
<i>N. testaceicornis</i> vs <i>T. spinipes</i>	1.0	< 0.0021	Nonacosene ^{*b} , <i>n</i> -C ₂₇ , <i>n</i> -C ₂₅
<i>N. testaceicornis</i> vs <i>T. angustula</i>	1.0	< 0.0021	Nonacosene ^{*b} , <i>n</i> -C ₂₅
<i>M. subnitida</i> vs <i>T. spinipes</i>	1.0	< 0.0021	<i>n</i> -C ₂₇ , Heptacosene ^{*b}
<i>M. subnitida</i> vs <i>T. angustula</i>	1.0	< 0.0021	Nonacosene ^{*b} , <i>n</i> -C ₂₅ , <i>n</i> -C ₂₇
<i>M. subnitida</i> vs <i>S. depilis</i>	0.902	< 0.0021	Heptacosene ^{*a} , Nonacosene ^{*b} , Hentriacontene ^{*c}
<i>T. spinipes</i> vs <i>T. angustula</i>	0.9633	< 0.0021	<i>n</i> -C ₂₅ , <i>n</i> -C ₂₇ , <i>n</i> -C ₃₁
<i>T. spinipes</i> vs <i>S. depilis</i>	0.9998	< 0.0042	<i>n</i> -C ₂₇ , Heptacosene ^{*a} , Heptacosene ^{*b}
<i>T. angustula</i> vs <i>S. depilis</i>	0.9989	< 0.0021	<i>n</i> -C ₂₅ , Heptacosene ^{*a} , <i>n</i> -C ₂₇ , Nonacosene ^{*b}

R values between 0 and 1 indicate the level of similarity or dissimilarity ($R = 0$ indicates no difference between the species, $R = 1$ indicates a larger similarity within a group than between groups), $P =$ Bonferroni adjusted P -values. * Different positions of double bonds (not identified).

common to almost all social insects and their functions seem to be more related to protection against desiccation, damage, and predators than having any function as a likely aggregation pheromone (Blomquist et al. 1998, Ayasse et al. 2001, Provost et al. 2008, Leonhardt et al. 2013).

To date, very little is known about the ability of male stingless bees to emit recruitment (aggregation) or sex pheromones and, thus, to attract partners for aggregations, albeit it has been suggested (Sommeijer et al. 2004, López and Kraus 2009). But, although none chemical experiment for attraction has been done here, our analysis of CHC profiles of different stingless bee species indicated that this is, as a whole, unlikely to explain the uncommon sexual behavior assumed by alien males. Future analysis testing behavioral responses between alien and host male odors may help us to better explain this uncommon behavior. We further suggest here another study possibility.

For example, it is known that *Trigonidium obtusum* and *Mormolyca ringens* orchids (Neotropical plant species) are chemically capable of mimicking some compounds (e.g., 2-alcohols and 9-alkene/alkane series) found in the sex queen pheromones of the stingless bees *N. testaceicornis*, *P. droryana*, and *Scaptotrigona* sp. attracting, then, these males for pseudocopulation (Singer 2002, Singer et al. 2004, Flach et al. 2006). Males of these stingless bee species, mentioned here, were already observed previously in mixed male aggregations (Kerr et al. 1962, Santos et al., 2014, Imperatriz-Fonseca apud Cortopassi-Laurino, 1979). Thus, sex queen pheromone of multiple stingless bee species could also be investigated by looking to see whether such pheromones could have any role in attracting different stingless bee males like have been observed for drones of different *Apis* species (Butler et al. 1967, Sannasi and Rajulu 1971, reviewed by Koeniger and Koeniger 2000).

Although cues causing attraction of alien males for reproductive aggregations of other stingless bee species are still unclear, this unexpected behavior may have deep implications for individual fitness of such males, as well as for its origin colonies. First, reproductive aggregations in stingless bees do not regularly occur throughout the year (Kerr et al. 1962, Cortopassi-Laurino 2007, Bänziger and Khamyotchai 2014, Santos et al. 2014). Therefore, males have few alternatives to choose correct aggregations to join during its short lifetime outside nests (2–3 wk). Second, although reproductive aggregations have thousands of individuals (Kerr et al. 1962, Santos et al. 2013, Bänziger and Khamyotchai 2014), the colonies usually send few males, oftentimes

just one, for every aggregation (Paxton 2000, Cameron et al. 2004). It means that if such males adopt any mistaken behavior during this lifetime, it may not only compromise its own reproductive success estimated to be in the order of 0.01–0.002 (Velthuis et al. 2005) but also it to reduce still more chances of colonies (ultimately its mother queens) pass genes forward.

Stingless bee males are under strong sexual selection to find queens and copulate with them (Roubik 1990). The reproductive aggregations are extremely male-biased (Kerr et al. 1962, Cortopassi-Laurino 2007, Bänziger and Khamyotchai 2014, Santos et al. 2014) and only one can mate successfully (Kerr et al. 1962, Strassmann 2001, Jaffé et al. 2014). Therefore it is likely that morphological, physiological, and behavioral features should maximize the competitive ability and the male's capacity to find and join conspecific aggregations. Nevertheless, alien stingless male bees seem to have lost their selective ability by adopting an apparently nonadaptive behavior. Furthermore, studies are needed to clarify this issue.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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