ORIGINAL ARTICLE

Relatedness and dispersal distance of eusocial bee males on mating swarms

Charles Fernando dos SANTOS[1], Vera Lucia IMPERATRIZ-FONSECA[2] and Maria Cristina ARIAS[3]

[1] *Departamento de Biodiversidade e Ecologia, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil*, [2] *Laboratório de Abelhas, Departamento de Ecologia, IB, Universidade de São Paulo, São Paulo, Brazil and* [3] *Departamento de Genética e Biologia Evolutiva, IB, Universidade de São Paulo, São Paulo, Brazil*

Abstract

Stingless bee males (Hymenoptera: Apidae) aggregate themselves for reproductive purposes. The knowledge of relatedness among the males attending the aggregations and the distance that they disperse from their natal nests to aggregations may provide important data to effectively conserve these bees. Here, we estimated these properties for *Tetragonisca angustula* (Latreille, 1811) males. Microsatellite molecular markers were used to genotype bees sampled from local nests and in mating swarms in order to identify the nests of origin of males and maternal genotypes of concerning queens. The distances from assigned nests to the mating swarms allowed us to estimate the distances travelled by males. A genetic relationship analysis was conducted to verify whether *T. angustula* males were closely related to nests where they aggregated. A pairwise relatedness analysis was also performed among all *T. angustula* males in each mating swarm. Our results demonstrated that *T. angustula* mating swarms received dozens to hundreds of males from several colonies (up to 70). Only two of the five mating swarms contained any males that were closely related to the bees from the new nests in construction. The relatedness among males was also extremely low. Yet, dispersal distance of *T. angustula* males ranged hundreds of meters up to 1.6 km, with evidence of reaching 2.25 km according to their flight radius obtained from their foraging area for locality. These data indicate a highly efficient mating system with minimal inbreeding in this bee species, with a great dispersal capability not previously found for stingless bee males.

Key words: Apidae, flight range, Hymenoptera, kinship, social insect.

INTRODUCTION

Clustering of one sex, generally males, in order to intercept or attract the opposite sex for reproductive purposes is common throughout the animal kingdom (Emlen & Oring 1977). This phenomenon occurs in marine and terrestrial environments, and in phylogenetically diverse animal groups such as fish, mammals, lizards and birds (Taylor *et al.* 1998; Heindl & Winkler 2003; Bro-Jørgensen 2007; Vitousek *et al.*

2007). In invertebrates such aggregations are routinely observed in insects, e.g. mayflies, damselflies, butterflies and flies (Thornhill & Alcock 1983). Similarly, in hymenopterans (ants, bees and wasps) large aggregations of males, or mating swarms (MS), have been described (Alcock *et al.* 1978; Boomsma *et al.* 2005; Paxton 2005).

Particularly in bees, male aggregations have mainly been studied in honeybee *Apis mellifera* (Linnaeus, 1758) (Apini). The drone congregation areas (DCA) are located high above ground on aerial landscapes (Alcock *et al.* 1978; Koeniger *et al.* 2005a; Paxton 2005). The honeybee virgin queens fly out seeking DCAs. Once in a DCA, the queens promptly release sex pheromones attracting drones to copulate (Alcock *et al.* 1978; Koeniger *et al.* 2005a; Paxton 2005). Yet, bumblebees (Bombini) and stingless bees (Meliponini) show more than one reproductive strategy. Bumblebee males may aggregate

Correspondence: Charles Fernando dos Santos, Departamento de Biodiversidade e Ecologia, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil.
Email: charles.santos@pucrs.br

and wait for virgin queens on a perch or at a hilltop, or patrol scent-marked routes (Bergman & Bergstrom 1997; Hovorka *et al.* 1998; Goulson *et al.* 2011). Also, they may aggregate near nests where a virgin queen lies, as observed for *Bombus muscorum* (Darvill *et al.* 2007). In tropical and subtropical stingless bees it has been demonstrated that males always aggregate. The aggregations can be located near mature nests, or nests being founded by workers on tree trunks and on artificial substrates (Michener 1946; Kerr *et al.* 1962; Roubik 1990; Cortopassi-Laurino 2007; Santos *et al.* 2014, 2015). The different male strategies are shown in Figure S1.

In general, the stingless bee virgin queen flies through the MS and is promptly chased by most males present there (Kerr *et al.* 1962; Engels & Imperatriz-Fonseca 1990). But, differently from honeybee, which is well known for extreme polyandry (Tarpy & Page 2000; Schlüns *et al.* 2005), the stingless bee virgin queen usually copulates with one male (monoandry) according to observation and molecular data (Kerr *et al.* 1962; Strassmann 2001; Jaffé *et al.* 2014).

In Hymenoptera, monoandry may not be an effective mating system, especially if related individuals mate. Sex determination in bees is affected by inbreeding and is controlled by a combination of two genetic systems: the arrhenotokous haplodiploidy and the complementary sex determination loci (CSD). Females arise from fertilized eggs and are heterozygous at the CSD, while males arise from unfertilized eggs and are hemizygous at the CSD loci. However, diploid individuals homozygous at the CSD loci result in diploid males (Whiting 1927; Cook & Crozier 1995). The presence of diploid males is generally associated with monoandrous inbred matings (Camargo 1979; Duchateau *et al.* 1994). The reproductive success of diploid males is thought to be practically nil because they are either functionally sterile or their offspring are triploid and nonviable (Camargo 1979; Duchateau *et al.* 1994; Darvill *et al.* 2012). Therefore, diploid males impose a high genetic load on the reproductive success of their colonies and, consequently, to the bee population at large (Zayed & Packer 2005).

Stingless bees are one of the most specious social bee groups (Michener 1969). Their MSs are large (Kerr *et al.* 1962; Santos *et al.* 2014) and include many unrelated males from several colonies, thus ensuring a high genetic diversity within the swarm (Paxton 2000; Cameron *et al.* 2004; Kraus *et al.* 2008; Mueller *et al.* 2012). To achieve this genetic diversity either the local density of colonies must be very high, or the MS must be able to recruit males from distant areas.

This first hypothesis appears unlikely since the colony density of stingless bees is generally relatively low, i.e.

0.01–0.42 nests/ha (Hubbell & Johnson 1977; Kajobe & Roubik 2006; Slaa 2006). It is therefore more plausible that males travel great distances from their maternal colony to join the MS. To date, it is known that the potential flight distance of males of *Scaptotrigona postica* (Latreille, 1807), *Melipona compressipes fasciculata* Smith, 1854 and *Melipona scutellaris* Latreille, 1811 may be at least 600–1000 m (Kerr *et al.* 1962; Carvalho-Zilse & Kerr 2004). Nonetheless, these experiments do not provide evidence for the true dispersal capability of males from their natal nest, since the individuals studied were captured in an MS, released from up to 600–1000 m away and then captured in the MS again.

Stingless bee males leave their natal colonies after reaching sexual maturity (two to three weeks old) and never return (Sakagami 1982). It seems quite likely that they may disperse over considerable distances until they find an MS.

Here we present data showing the dispersal distances of *Tetragonisca angustula* (Latreille, 1811) males using molecular markers. We were also able to estimate the number of colonies represented in an MS, and evaluated male relatedness within an MS and whether males were somewhat closely related to founding nests where they aggregated. This species is an ideal model as males frequently gather in the MS throughout the year near colonies with virgin queens or at new nests in the process of being built by workers (Kerr *et al.* 1962; Nogueira-Ferreira & Soares 1998; van Veen & Sommeijer 2000). This species has high priority for ecological services in South America. It has been demonstrated that *T. angustula* visits a large range of native flowers (e.g. Braga *et al.* 2012) and is an effective pollinator of commercial crops (Slaa *et al.* 2006).

MATERIALS AND METHODS

Study site, trap-nests and male swarms

This study was carried out on the campus of the Universidade de São Paulo, Ribeirão Preto, Brazil (USP-RP), with a total area of approximately 586 ha, during 2011. Ninety trap-nests based on Oliveira *et al.* (2012) were randomly lodged in trees at a height of 1 m. The trap-nests simulate natural hollows (e.g. in trees) and enable stingless bee workers to found new nests. Moreover, the nests in foundation attract male swarms.

Local nests and sampling of bees

A large survey of local nests of *T. angustula* in the study area was previously carried out by consulting a database (A. E. E. Soares, unpubl. data) of Meliponini nests on

the campus of USP-RP. The geographic coordinates of the local nests were recorded and at least five *T. angustula* guard workers were sampled from each nest. Also five workers from the trap-nests under occupancy were collected, as well as all the aggregated males flying in front of them. All these bees were immediately preserved in 99% ethanol during fieldwork and later kept at –20°C until DNA extraction.

DNA extraction and microsatellite genotyping

In the laboratory, bees were dried before DNA extraction. The thorax was removed and macerated in 400 μL of 10% Chelex (Walsh *et al.* 1991) with modifications (Francisco *et al.* 2001). Six microsatellite loci (Tang03, Tang11, Tang12, Tang57, Tang60 and Tang65) were used for genotyping (Brito *et al.* 2009). We followed the procedure described by Schuelke (2000), where each forward primer is synthesized with an additional sequence at the 5′end derived from M13 (5'-TGTAAAACGACGGCCAGT-3'). The polymerase chain reaction (PCR) was performed in 5 μL final volume containing: 2.35 μL autoclaved Milli-Q H_2O, 0.5 μL buffer PCR 10 × (Invitrogen, Waltham, MA, USA), 0.5 μL dNTPs (2 mM each), 0.2 μL $MgCl_2$ (50 mM), 0.125 μL M13 primer labeled with 6-HEX, FAM or NED (5 μM), 0.125 μL of forward and reverse primers mix (F-modified 0.5 μM + R 5 μM), 0.1 μL of glycerol 50%, 0.1 μL *Taq* DNA polymerase (Invitrogen) 5U/μL and 1 μL of DNA template from Chelex extraction. PCR reaction conditions for each locus are presented in Francisco *et al.* (2014).

PCR products were electrophoresed in an automated sequencer (ABI 3730 DNA Analyser; Applied Biosystems, Waltham, MA, USA) and GeneScan 500 ROX (Applied Biosystems) was used as a molecular weight marker. The software GENEMAKER 2.2 (Pittsburgh, PA, USA) was applied for genotyping.

Population genetic analyses

The following population parameters were estimated: (i) number of alleles per locus; (ii) expected heterozygosity (H_e); and (iii) linkage disequilibrium. The program GENEPOP 4.1.4 (Raymond & Rousset 1995) was used to perform the analyses. Others population parameters as population differentiation and structure have been reported elsewhere (Santos *et al.* 2016).

Colony assignment

Both male and worker genotypes from local nests and trap-nests were used to reconstruct sibship and to infer males' natal nests using the program COLONY 1.2

(Wang 2004). This program estimates the genetic relationship between haplodiploidy individuals by maximum likelihood, inferring their putative families based on population allele frequency (Wang 2004). Thus, males could be assigned to specific local nests.

The number of colonies contributing males was estimated by analyzing the output of COLONY. The accuracy of the COLONY algorithm has generally been considered to be relatively high (Harrison *et al.* 2013) and other studies have successfully used COLONY to the same purpose as here (see Discussion). Genotyping error and mutation rate were included in the analysis with values of 0.05 each. Three independent runs were performed with different seeds and the highest log-likelihood converging yields (mother colonies) were used for reconstruction by consensus.

Estimating male-contributing colonies using molecular markers is subject to non-sampling errors (NSE); i.e. a number of colonies may remain undetected because of insufficient sampling effort. Thus, NSE was evaluated by using a fitted Poisson distribution and calculating the expected frequency for categories of zero or less than one (Chapman *et al.* 2003). NSE was estimated using R (R Core Team 2015) by using a goodness-of-fit test for the Poisson distribution calculated by maximum likelihood method using the goodfit function in the *vcd* package (Meyer *et al.* 2015).

Maternal genotype and relatedness between bees

The putative maternal genotypes were estimated by using genotypes of *T. angustula* males caught in an MS and of workers from local and trap-nests in COLONY 1.2. The most likely maternal genotypes in the COLONY (highest log probability) were used in our analysis.

To estimate the relatedness (r) between males caught in a given MS and queens concerning (i.e. inferred genotypes) to the nests where males aggregated we used the KINGROUP 2 program (Konovalov *et al.* 2004). First, an analysis of pairwise relatedness of the males in every MS was performed using the Queller and Goodnight (1989) estimator in KINGROUP 2 (Konovalov *et al.* 2004). The *r* values ranged from –1 to +1. A positive value indicates that two individuals share more alleles by descent than expected by chance. Unrelated individuals should have an *r* value equal to 0 (Queller & Goodnight 1989). When *r* is negative in pairwise comparisons it implies that the individuals concerned are more related to any other individual in a population than they are to themselves (Konovalov & Heg 2008).

It was then tested whether males were closely related to the nests concerned using the likelihood ratio test (LRT) in pedigree relationships in KINGROUP 2 (Konovalov *et al.*

2004). It estimates the probability of sharing identical alleles by descent (Goodnight & Queller 1999). The simulation was performed for 20 000 pairings between *T. angustula* males and the queens concerned. The assignment of closely related individuals was accepted at $P \leq 0.01$.

Dispersal distance

After the assignment of males' natal nests using COLONY, we used a global positioning system (GPS) device to estimate geographic distance travelled by the males between natal nest (those previously surveyed) and the MS where they were collected. Data were analyzed using the GPS TRACKMAKER 13.8 program.

As data showed a distribution of parabolic curve type (*post-hoc* analysis), we then evaluated these data by using a second-order polynomial regression analysis assuming ordinary least squares and fixed x values to find a model fit that minimizes the square errors in the y values. This analysis uses a second-order polynomial function defined as $y = ß_0 + ß_1 x + ß_2 x^2 + e$, where y is the outcome variable, $ß_0$ is the intercept parameter, $ß_1$ is the linear effect parameter, $ß_2$ is the quadratic effect parameter, x is the independent (predictor) variable, i.e. dispersal distance, and e is the related random error. That analysis was carried out by adapting the quadratic model in the *lm* function of the *stats* package (R Core Team 2015).

Additionally, we estimated the flight range of *T. angustula* males by slightly modifying approaches of Kraus *et al.* (2009) and Wolf *et al.* (2012). The analysis was based on the colony density in the study area (see Results). We then evaluated the relative frequency of male-contributing colonies localized within the study area to estimate the overall number of nests. The foraging area of *T. angustula* males was estimated according to Equation 1:

$$FA_{males} = \frac{nests}{area} \tag{1}$$

where FA_{males} is the male foraging area, *nests* is the number of male-donating nests estimated from frequency of assigned colonies according to COLONY and *area* is

the nest density estimated within the study area. The shape was assumed to be circular and the pattern measure used was km^2.

The *T. angustula* male flight radius (FR_{males}) was calculated from Equation 2:

$$FR_{males} = \sqrt{\frac{FA_{males}}{\pi}} \tag{2}$$

All data were visualized using *ggplot2* (Wickham 2009) and a suitable *ggmap* (Kahle & Wickham 2013) package built for R (R Core Team 2015).

RESULTS

Natural nests and studied mating swarms

In total 76 natural nests of *T. angustula* were located in the study area representing a colony density of 0.12 nests/ha (i.e. 12 nests/km^2). Only five out of 90 installed trap-nests (Fig. S2) attracted *T. angustula* MS in a relatively short period, i.e. within 34 days. All together, 378 males were sampled from these MSs (Table 1).

Parameters of genetic diversity

The number of alleles per locus varied from seven to 28, the average was 9.8 ± 0.901 ($\pm$ SD) and the effective number of alleles was 5.2 ± 0.613 ($\pm$ SD) (Table 2).

The expected heterozygosity was high (Table 2) and linkage disequilibrium between loci was not verified ($P > 0.05$), and so all loci were mutually independent. Two diploid males were sampled at one mating swarm, and this finding has been reported elsewhere (Santos *et al.* 2013).

Male-contributing colonies and males per colony

Overall, 83 colonies were estimated to have donated males to MSs (Tables 1, S3). The number of non-sampled colonies calculated by truncated Poisson distribution was very low ($\chi^2 = 7.006$, *d.f.* = 11, $P > 0.5$), resulting in an estimated NSE of 0.87 non-sampled colonies. It demonstrates that a large majority of nests within the

Table 1 Number of *Tetragonisca angustula* males per mating swarm, number of male-contributing colonies and relatedness among males in each mating swarm

	Mating swarms					
	A	B	C	D	E	Total
Number of males	57	99	56	16	150	378
Number of assigned colonies	36	59	42	14	70	83[†]
Global pairwise relatedness (mean ± SD)	−0.018 ± 0.20	−0.010 ± 0.20	−0.017 ± 0.21	−0.066 ± 0.20	−0.007 ± 0.20	−0.0097 ± 0.20

[†]Many males assigned to the same colony (i.e. brothers) were often found in different mating swarms.

Table 2 Total number of alleles per locus observed in *Tetragonisca angustula* males and expected heterozygosity estimated for each mating swarm

Locus	Tang57	Tang03	Tang12	Tang11	Tang65	Tang60
Alleles by locus	13	7	13	16	28	11
Mating swarms			Expected heterozygosity (H_e)			
A	0.317	0.707	0.879	0.718	0.939	0.809
B	0.357	0.687	0.889	0.759	0.922	0.803
C	0.375	0.496	0.885	0.647	0.923	0.805
D	0.608	0.675	0.883	0.905	0.925	0.650
E	0.413	0.661	0.841	0.752	0.930	0.819

study area was effectively surveyed. On average each colony released 4.6 ± 2.86 (± SD) males (Fig. 1), although each MS has usually recruited just one or two *T. angustula* males per colony (Table S3).

Genetic relationship among bees

The global relatedness among all males was estimated to be extremely low ($r = -0.0097 \pm 0.20$, mean ± SD), as well as the relatedness among males within each MS (Table 1). We found that only nine males out of 378 (i.e. 2.38%) were closely related to the concerning virgin queens from the trap-nest where they aggregated (LRT = 12.091, $P < 0.01$; Fig. 2, Table 3).

Dispersal distance of males

One hundred and thirty males were successfully assigned to 33 previously surveyed nests at the locality (Table S3). By comparing the geographical coordinates between those 33 nests and the MS where males were collected, we estimated that dispersal distance was on average 612 ± 434 (± SD) m. The minimum distance was 10 m, while the maximum

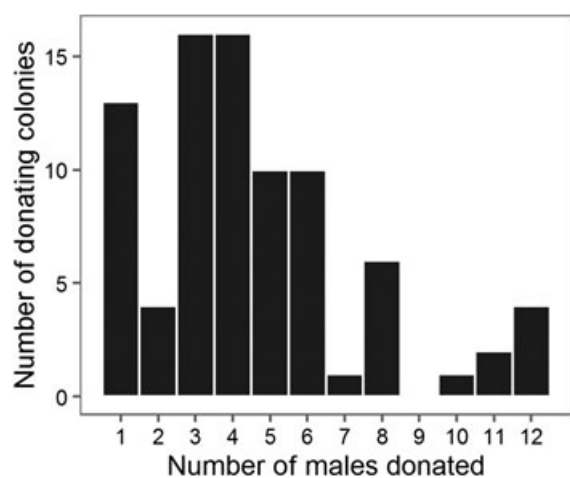**Figure 1** Number of *Tetragonisca angustula* colonies *versus* number of males donated.

distance was 1677 m (Fig. 3). Yet, the number of *T. angustula* males was significantly affected by distance from their mother nests to the MSs ($F = 15.74$, $d.f. = 8$, adjusted $R^2 = 0.74$, $P < 0.001$; Fig. 3). The residual mean-square error was 2.921 with 8 degrees of freedom. The spatial distribution of assigned colonies that donated *T. angustula* males for a certain MS are shown in Figure 4.

Finally, after having verified that *T. angustula* males came from 33 colonies (i.e. 43% from 76 surveyed nests at the locality), we estimated that male's foraging area should be larger than the study area and should hold at least 193 *T. angustula* nests. Thus, by applying equation 1 we estimated the male's foraging area to be 16 km² and converted (Eqn 2) it to the male flight distance capacity (radius) of 2.25 km.

DISCUSSION

Relatedness and amount of colonies

Our findings demonstrated that *T. angustula* males in mating swarms were genetically less related either to one another or to the nests where they aggregated. We found that males came from several colonies. Our work therefore supports previous studies that verified the low relatedness among male bees near the nests where they aggregated. For instances, males of the Asian stingless bee *Trigona collina* (Smith, 1857) and the bumblebee *Bombus muscorum* (Linnaeus, 1758) have a similar tendency to avoid congregating in front of closely related nests (Cameron *et al*. 2004; Darvill *et al*. 2007). Our data support previous work indicating that the mating system widely observed in stingless bees may contribute to reducing chances of inbreeding (Paxton 2000; Cameron *et al*. 2004; Kraus *et al*. 2008; Mueller *et al*. 2012). Furthermore, this phenomenon seems to be fairly common in other social bees such as honeybee and bumblebee species (Baudry *et al*. 1998; Kraus *et al*. 2005; Darvill *et al*. 2007; Jaffé & Moritz 2010).
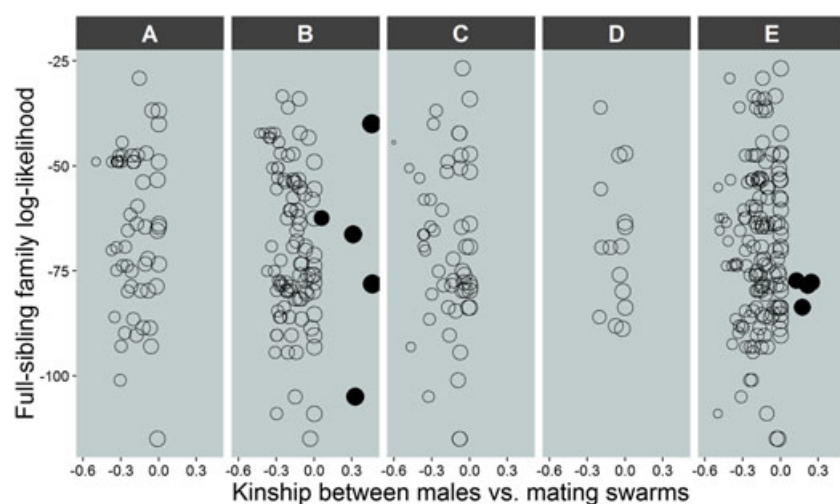
Figure 2 Genetic relatedness and assignment test for males; *x*-axis, genetic relatedness between *Tetragonisca angustula* males from the same mating swarms (KINGROUP program) for each of five different swarms (A–E). Larger circles denote higher pairwise relatedness (positive scores) between males and colonies where they were aggregated; black circles denote the males more closely related to the virgin queen from the nest in front of which they aggregated. *y*-axis: full-sibling family log-likelihood denoting the most parsimonious configuration assigning males to their natal colonies (COLONY program); in other words, males shown exactly on the same horizontal line (equal maximum log-likelihood score) belong to the same family (natal colony).

Table 3 Genotypes represented by allele code(s) of *Tetragonisca angustula* queens (diploid) and males (haploid) concerned with mating swarms

Queens[†]	Locus					
	Tang57	Tang03	Tang12	Tang11	Tang65	Tang60
A	184/186	189/211	215/223	184/188	192/226	147/147
B	156/186	219/219	217/227	180/184	210/224	141/147
C	156/194	217/219	207/217	196/200	192/226	141/149
D	184/184	209/219	217/227	180/184	190/192	147/147
E	156/200	219/219	199/217	186/194	220/220	145/147

Males[‡] (code No.)	Locus						Relatedness (*r*) male ↔ queen	Pedigree relationship
	Tang57	Tang03	Tang12	Tang11	Tang65	Tang60		
99[§]	194	219	221	206	208	145	0.0061	<0.01
101[§]	156	219	217	184	196	153	0.3113	<0.01
106[§]	156	219	217	184	224	153	0.4640	<0.01
131[§]	156	217	217	184	224	141	0.3271	<0.01
133[§]	156	219	217	184	224	139	0.4603	<0.01
235[#]	156	221	217	182	200	149	0.1744	<0.01
268[#]	156	219	207	182	222	143	0.2447	<0.01
277[#]	194	221	217	204	202	141	0.2178	<0.01
279[#]	200	219	221	–	220	143	0.1259	<0.01

[†]Genotypes of mother queens were inferred from workers in nest-traps where mating swarms (A–E) occurred.
[‡]Males genetically close to the queens concerned with swarms.
[§]Individuals collected onto swarm B.
[#]Individuals collected onto swarm E.
–, not amplified.

Therefore, although the competition among males is high in an MS, the queen gets the benefit of decreasing the chance of mating with closely related partner. This may greatly contribute to reducing the inbreeding risk, which brings a high cost of diploid male production to colonies of these social bees. Diploid males are usually cannibalized (honeybees), attacked by workers (stingless bees) or low in viability (bumblebees), but sometimes they produce a triploid brood (Duchateau *et al.* 1994; Santomauro & Oldham 2004; Darvill *et al.* 2012; Francini *et al.* 2012). However, some males can escape the workers' harassment (e.g. stingless bees) and visit the MS (Santos *et al.* 2013; Vollet-Neto *et al.* 2015)

We observed that each nest donated on average 4.6 males to several MSs. This phenomenon of brothers
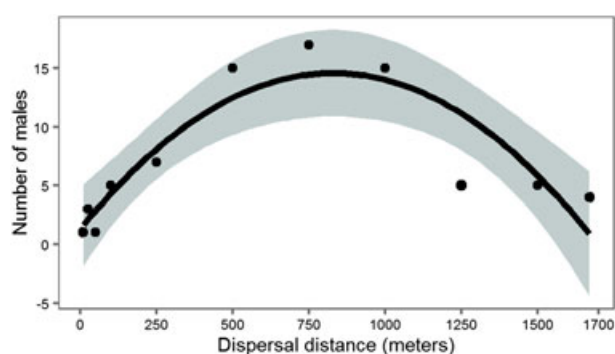
Figure 3 Dispersal distances of *Tetragonisca angustula* males from their natal nests to mating swarms. Quadratic regression: black line, 2nd order polynomial function; black points, means; shaded, 95% confidence interval.

attending different MSs has been also observed in other stingless bee, honeybee and bumblebee species (Baudry *et al*. 1998; Cameron *et al*. 2004; Darvill *et al*. 2007; Kraus *et al*. 2008; Jaffé & Moritz 2010; Mueller *et al*. 2012). This dispersing behavior is usually more expected in species where males never return to their mother nests, like in stingless bees and bumblebees (e.g. *B. muscorum*). In honeybees, males return to their mother colony after joining drone congregations during the day time (Koeniger *et al*. 2005b), but it has been verified that male congregations include a great proportion of singletons, i.e. a single male from the same nest (Baudry *et al*. 1998; Collet *et al*. 2009).

The finding that *T. angustula* males from the same colony have been sampled in different MSs suggests that they may disperse randomly after leaving their natal nests. Thus, although there may be a strong competition among distantly related males in an MS, there is less competition between brothers and thereby a potential increase in the reproductive fitness of their natal colony if such individuals successfully mate.

It is interesting that not all colonies located in the study area donated males during the time of this survey. We verified that at least 33 (= 76 – 43) local colonies

contributed as male donors to MSs. This is to say that out of all 83 male-donating colonies identified by our genetic analysis, about 50 of them most likely were outside our study area.

Dispersal distance

How exactly we can detect distribution of brothers over different male aggregations is still an open question even in honeybees. It has been suggested that *A. mellifera* drones either stay at the first aggregation they have reached, or they visit several aggregations, compare them and select the most attractive one (Koeniger *et al*. 2005b). How *T. angustula* males behave needs to be investigated further. But, if we assume that *T. angustula* males visit several aggregations until the final choice, we should expect their high dispersal from natal nests to cover the whole environment searching for the best aggregation. Our estimation indicates that males disperse, on average, about 600 m from their natal colonies to MSs.

Moreover we verified that some *T. angustula* males dispersed up to 1.67 km (GPS data), and according to our estimates using the radius of potential foraging area they may even travel 2.25 km. These distances are far greater than previously described for other species of stingless bees (Kerr *et al*. 1962; Carvalho-Zilse & Kerr 2004). Such a great dispersal capability as 1–5 km was also reported in males of other social hymenopterans, e.g. *Eciton burchellii* Westwood, 1842, *Bombus terrestris* (Linnaeus, 1758), *B. lapidarius* (Linnaeus, 1758) and *A. mellifera*. This great male dispersal may largely contribute to gene flow among populations (Ruttner & Ruttner 1972; Jaffé *et al*. 2009; Kraus *et al*. 2009; Wolf *et al*. 2012).

Mueller *et al*. (2012) suggested two methods for estimating the flight range of male stingless bees. One method is marking and recapture of males and measuring the distance they have travelled, whereas the other is estimating the local colony density based on the number of colonies found in a given area. Here we presented the third alternative method by georeferencing a great number
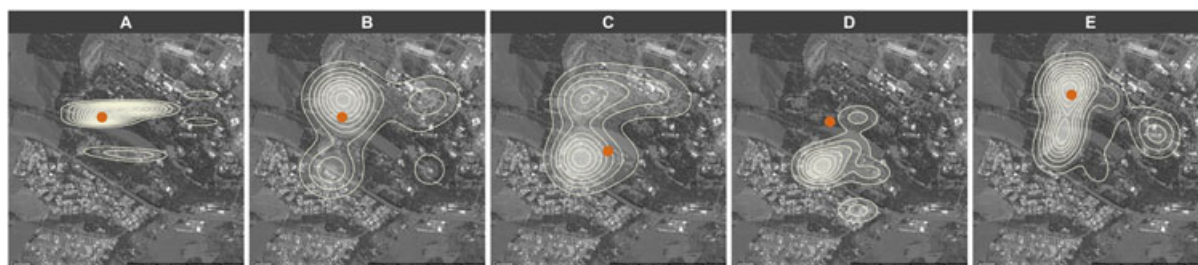
Figure 4 Contour plots of spatial distribution of assigned colonies (white lines) donating *Tetragonisca angustula* males for five different mating swarms (A–E) (red points).

of local nests and genetically assigning males collected on MSs to maternal colonies. Thus, we estimated the travelled distance of *T. angustula* males to be 1.67 km at maximum. Additionally, we also applied the second method of Mueller *et al.* (2012), as in Kraus *et al.* (2009) and Wolf *et al.* (2012), to estimate the potential flight distance of *T. angustula* males, and inferred that it was larger, i.e. 2.25 km, than the above estimate.

It would be of interest to investigate whether the significant dispersal of males, as shown here, also plays an important role in promoting the genetic diversity of plants. However, the importance of stingless bee males as pollinators has been neglected despite their presence on flowers (Kerr *et al.* 1962; Sommeijer *et al.* 2004), in contrast to males of solitary and other social bees, such as *Colletes cunicularius* Latreille, 1802, *B. terrestris* and *B. lapidarius*, which also fly over substantial distances and contribute to pollination to more or less extent (Peakall & Schiestl 2004; Kraus *et al.* 2009; Wolf *et al.* 2012; Wolf & Moritz 2014).

In conclusion, our study demonstrates that: (i) a *T. angustula* MS is composed of males from multiple colonies; (ii) brothers tend to join different MSs; (iii) this behavior leads to a low relatedness among the members of a MS; and (iv) males disperse over considerable distances and are often found in MSs far from their natal nests. Together, these findings suggest a highly effective mating system that reduces the chances of mating between closely related individuals.

ACKNOWLEDGMENTS

REFERENCES

Alcock J, Barrows EM, Gordh G *et al.* (1978) The ecology and evolution of male reproductive behaviour in the bees and wasps. *Zoological Journal of the Linnean Society* **64**, 295–326.

Baudry E, Solignac M, Garnery L, Gries M, Cornuet J-M, Koeniger N (1998) Relatedness among honeybees (*Apis mellifera*) of a drone congregation. *Proceedings of the Royal Society B: Biological Sciences* **265**, 2009–2014.

Bergman P, Bergstrom G (1997) Scent marking, scent origin, and species specificity in male premating behavior of two Scandinavian bumblebees. *Journal of Chemical Ecology* **23**, 1235–1251.

Boomsma JJ, Baer B, Heinze J (2005) The evolution of male traits in social insects. *Annual Review of Entomology* **50**, 395–420.

Braga JA, Sales EO, Neto JS, Conde MM, Barth OM, Lorenzon MC (2012) Floral sources to *Tetragonisca angustula* (Hymenoptera: Apidae) and their pollen morphology in a Southeastern Brazilian Atlantic forest. *Revista de Biología Tropical* **60**, 1491–1501.

Brito RM, Francisco FO, Domingues-Yamada AMT *et al.* (2009) Characterization of microsatellite loci of *Tetragonisca angustula* (Hymenoptera, Apidae, Meliponini). *Conservation Genetics Resources* **1**, 183–187.

Bro-Jørgensen J (2007) Reversed sexual conflict in a promiscuous antelope. *Current Biology* **17**, 2157–2161.

Camargo CA (1979) Sex determination in bees. XI. Production of diploid males and sex determination in *Melipona quadrifasciata* (Hym., Apidae). *Journal of Hymenoptera Research* **18**, 77–84.

Cameron EC, Franck P, Oldroyd BP (2004) Genetic structure of nest aggregations and drone congregations of the southeast Asian stingless bee *Trigona collina*. *Molecular Ecology* **13**, 2357–2364.

Carvalho-Zilse GA, Kerr WE (2004) Natural substitutions of queens and flight distance of males in tiuba (*Melipona compressipes fasciculata* Smith, 1854) and uruçu (*Melipona scutellaris* Latreille, 1811) (Apidae, Meliponini). *Acta Amazonica* **34**, 649–652. (In Portuguese.)

Chapman RE, Wang J, Bourke AFG (2003) Genetic analysis of spatial foraging patterns and resource sharing in bumble bee pollinators. *Molecular Ecology* **12**, 2801–2808.

Collet T, Cristino AS, Quiroga CFP, Soares AEE, Del Lama MA (2009) Genetic structure of drone congregation areas of Africanized honeybees in southern Brazil. *Genetics and Molecular Biology* **32**, 857–863.

Cook JM, Crozier RH (1995) Sex determination and population biology in the Hymenoptera. *Trends in Ecology & Evolution* **10**, 281–286.

Cortopassi-Laurino M (2007) Drone congregations in Meliponini: what do they tell us? *Bioscience Journal* **23**, 153–160.

Darvill B, Lye GC, Goulson D (2007) Aggregations of male *Bombus muscorum* (Hymenoptera: Apidae) at mature nests. Incestuous brothers or amorous suitors? *Apidologie* **38**, 518–524.

Darvill B, Lepais O, Woodall LC, Goulson D (2012) Triploid bumblebees indicate a direct cost of inbreeding in fragmented populations. *Molecular Ecology* **21**, 3988–3995.

Duchateau MJ, Hoshiba H, Velthuis HHW (1994) Diploid males in the bumble bee *Bombus terrestris*. *Entomologia Experimentalis et Applicata* **71**, 263–269.

Emlen ST, Oring LW (1977) Ecology, sexual selection, and the evolution of mating systems. *Science* **197**, 215–223.

Engels W, Imperatriz-Fonseca VL (1990) Caste development, reproductive strategies, and control of fertility in honey bees and stingless bees. In: Engels W (ed.) *Social Insects: An Evolutionary Approach to Castes and Reproduction*, pp 167–230. Springer, Berlin Heidelberg, Berlin.

Francini IB, Nunes-Silva CG, Carvalho-Zilse GA (2012) Diploid male production of two amazonian *Melipona* bees (Hymenoptera: Apidae). *Psyche* **2012**, Article ID 484618. DOI:10.1155/2012/484618

Francisco FO, Silvestre D, Arias MC (2001) Mitochondrial DNA characterization of five species of *Plebeia* (Apidae: Meliponini): RFLP and restriction maps. *Apidologie* **32**, 323–332.

Francisco FO, Santiago LR, Brito RM, Oldroyd BP, Arias MC (2014) Hybridization and asymmetric introgression between *Tetragonisca angustula* and *Tetragonisca fiebrigi*. *Apidologie* **45**, 1–9.

Goodnight KF, Queller DC (1999) Computer software for performing likelihood tests of pedigree relationship using genetic markers. *Molecular Ecology* **8**, 1231–1234.

Goulson D, Sangster EL, Young JC (2011) Evidence for hilltopping in bumblebees? *Ecological Entomology* **36**, 560–563.

Harrison HB, Saenz-Agudelo P, Planes S, Jones GP, Berumen ML (2013) Relative accuracy of three common methods of parentage analysis in natural populations. *Molecular Ecology* **22**, 1158–1170.

Heindl M, Winkler H (2003) Interacting effects of ambient light and plumage color patterns in displaying wire-tailed manakins (Aves, Pipridae). *Behavioral Ecology and Sociobiology* **53**, 153–162.

Hovorka O, Urbanová K, Valterová I (1998) Premating behavior of *Bombus confusus* males and analysis of their labial gland secretion. *Journal of Chemical Ecology* **24**, 183–193.

Hubbell SP, Johnson LK (1977) Competition and nest spacing in a tropical stingless bee community. *Ecology* **58**, 949–963.

Jaffé R, Moritz RFA (2010) Mating flights select for symmetry in honeybee drones (*Apis mellifera*). *Naturwissenschaften* **97**, 337–343.

Jaffé R, Moritz RFA, Kraus FB (2009) Gene flow is maintained by polyandry and male dispersal in the army ant *Eciton burchellii*. *Population Ecology* **51**, 227–236.

Jaffé R, Pioker-Hara FC, Santos CF *et al.* (2014) Monogamy in large bee societies: a stingless paradox. *Naturwissenschaften* **101**, 261–264.

Kahle D, Wickham H (2013) ggmap: spatial visualization with ggplot2. *The R Journal* **5**, 144–161.

Kajobe R, Roubik DW (2006) Honey-making bee colony abundance and predation by apes and humans in a Uganda Forest Reserve. *Biotropica* **38**, 210–218.

Kerr WE, Zucchi R, Nakadaira JT, Butolo JE (1962) Reproduction in the social bees (Hymenoptera: Apidae). *Journal of the New York Entomological Society* **70**, 265–276.

Koeniger N, Koeniger G, Gries M, Tingek S (2005a) Drone competition at drone congregation areas in four *Apis* species. *Apidologie* **36**, 211–221.

Koeniger N, Koeniger G, Pechhacker H (2005b) The nearer the better? Drones (*Apis mellifera*) prefer nearer drone congregation areas. *Insectes Sociaux* **52**, 31–35.

Konovalov DA, Heg D (2008) A maximum-likelihood relatedness estimator allowing for negative relatedness values. *Molecular Ecology Resources* **8**, 256–263.

Konovalov DA, Manning C, Henshaw MT (2004) Kingroup: a program for pedigree relationship reconstruction and kin group assignments using genetic markers. *Molecular Ecology Notes* **4**, 779–782.

Kraus FB, Koeniger N, Tingek S, Moritz RFA (2005) Temporal genetic structure of a drone congregation area of the giant Asian honeybee (*Apis dorsata*). *Naturwissenschaften* **92**, 578–581.

Kraus FB, Weinhold S, Moritz RFA (2008) Genetic structure of drone congregations of the stingless bee *Scaptotrigona mexicana*. *Insectes Sociaux* **55**, 22–27.

Kraus FB, Wolf S, Moritz RFA (2009) Male flight distance and population substructure in the bumblebee *Bombus terrestris*. *The Journal of Animal Ecology* **78**, 247–252.

Meyer D, Zeileis A, Hornik K (2015) *vcd: Visualizing Categorical Data* (R package version 1.4-1) [Computer software]. Available from URL: https://cran.r-project.org/package=vcd

Michener CD (1946) Notes on the habits of some Panamanian stingless bees (Hymenoptera, Apidae). *Journal of the New York Entomological Society* **54**, 179–197.

Michener CD (1969) Comparative social behavior of bees. *Annual Review of Entomology* **14**, 299–342.

Mueller MY, Moritz RFA, Kraus FB (2012) Outbreeding and lack of temporal genetic structure in a drone congregation of the neotropical stingless bee *Scaptotrigona mexicana*. *Ecology and Evolution* **2**, 1304–1311.

Nogueira-Ferreira FH, Soares AEE (1998) Male aggregations and mating flight in *Tetragonisca angustula*. *Iheringia, Série Zoologia* **84**, 141–144.

Oliveira RC, Menezes C, Soares AEE, Imperatriz- Fonseca VL (2012) Trap-nests for stingless bees (Hymenoptera, Meliponini). *Apidologie* **44**, 29–37.

Paxton RJ (2000) Genetic structure of colonies and a male aggregation in the stingless bee *Scaptotrigona postica*, as revealed by microsatellite analysis. *Insectes Sociaux* **47**, 63–69.

Paxton RJ (2005) Male mating behaviour and mating systems of bees: an overview. *Apidologie* **36**, 145–156.

Peakall R, Schiestl FP (2004) A mark-recapture study of male *Colletes cunicularius* bees: implications for pollination by sexual deception. *Behavioral Ecology and Sociobiology* **56**, 579–584.

Queller DC, Goodnight KF (1989) Estimating relatedness using genetic markers. *Evolution* **43**, 258–275.

R Core Team (2015) *R: A Language and Environment for Statistical Computing*. The R Foundation for Statistical Computing, Vienna. Available from URL: https://www.r-project.org/

Raymond M, Rousset F (1995) Genepop (version 1.2): Population genetics software for exact test and ecumenism. *The Journal of Heredity* **86**, 248–249.

Roubik DW (1990) Mate location and mate competition in males of stingless bees (Hymenoptera: Apidae: Meliponinae). *Entomologia Generalis* **15**, 115–120.

Ruttner H, Ruttner F (1972) Untersuchungen über die flugaktivität und das paarungsverhalten der drohnen. V. Drohnensammelplätze und paarungsdistanz. *Apidologie* **3**, 203–232.

Sakagami SF (1982) Stingless bees. In: Hermann HR (ed.) *Social Insects*, pp 361–423. Academic Press, New York.

Santomauro G, Oldham NJ (2004) Cannibalism of diploid drone larvae in the honey bee (*Apis mellifera*) is released by odd pattern of cuticular substances. *Journal of Apicultural Research* **43**, 69–74.

Santos CF, Menezes C, Imperatriz-Fonseca VL, Arias MC (2013) A scientific note on diploid males in a reproductive event of a eusocial bee. *Apidologie* **44**, 519–521.

Santos CF, Menezes C, Vollet-Neto A, Imperatriz-Fonseca VL (2014) Congregation sites and sleeping roost of male stingless bees (Hymenoptera: Apidae: Meliponini). *Sociobiology* **61**, 115–118.

Santos CF, Ferreira-Caliman MJ, Nascimento FS (2015) An alien in the group: eusocial male bees sharing nonspecific reproductive aggregations. *Journal of Insect Science* **15**, 157–163.

Santos CF, Francisco FO, Arias MC, Imperatriz-Fonseca VL (2016) Eusocial bee male aggregations: spatially and temporally separated but genetically homogenous. *Entomologia Experimentalis et Applicata* **158**, 320–326.

Schlüns H, Moritz RFA, Neumann P, Kryger P, Koeniger G (2005) Multiple nuptial flights, sperm transfer and the evolution of extreme polyandry in honeybee queens. *Animal Behaviour* **70**, 125–131.

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* **18**, 1–2.

Slaa EJ (2006) Spatial nesting patterns in a Neotropical stingless bee community: do bees compete for food? *Proceedings of the Netherlands Entomological Society Meeting* **17**, 71–78.

Slaa EJ, Sánchez Chaves LA, Malagodi-Braga KS, Hofstede FE (2006) Stingless bees in applied pollination: practice and perspectives. *Apidologie* **37**, 293–315.

Sommeijer MJ, Bruijn LLM, Meeuwsen F (2004) Behaviour of males, gynes and workers at drone congregation sites of the stingless bee *Melipona favosa* (Apidae: Meliponini). *Entomologsische Berichtein* **64**, 10–15.

Strassmann J (2001) The rarity of multiple mating by females in the social Hymenoptera. *Insectes Sociaux* **48**, 1–13.

Tarpy DR, Page RE Jr (2000) No behavioral control over mating frequency in queen honey bees (*Apis mellifera* L.): Implications for the evolution of extreme polyandry. *The American Naturalist* **155**, 820–827.

Taylor MI, Turner GF, Robinson RL, Stauffer JR jr (1998) Sexual selection, parasites and bower height skew in a bower-building cichlid fish. *Animal Behaviour* **56**, 379–384.

Thornhill R, Alcock J (1983) *The Evolution of Insect Mating Systems*. Harvard University Press, Cambridge, MA.

van Veen JW, Sommeijer MJ (2000) Colony reproduction in *Tetragonisca angustula* (Apidae, Meliponini). *Insectes Sociaux* **47**, 70–75.

Vitousek MN, Mitchell MA, Woakes AJ, Niemack MD, Wikelski M (2007) High costs of female choice in a lekking lizard. *PLoS ONE* **2**, Article ID e567. DOI:10.1371/journal.pone.0000567

Vollet-Neto A, Santos CF, Santiago LR *et al.* (2015) Diploid males of *Scaptotrigona depilis* are able to join reproductive aggregations (Apidae, Meliponini). *Journal of Hymenoptera Research* **45**, 125–130.

Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* **10**, 506–513.

Wang J (2004) Sibship reconstruction from genetic data with typing errors. *Genetics* **166**, 1963–1979.

Whiting A (1927) Genetic evidence for diploid males in Habrobracon. *The Biological Bulletin* **53**, 438–449.

Wickham H (2009) *ggplot2: Elegant Graphics for Data Analysis*. Springer, New York.

Wolf S, Moritz RFA (2014) The pollination potential of free-foraging bumblebee (*Bombus* spp.) males (Hymenoptera: Apidae). *Apidologie* **45**, 440–450.

Wolf S, Toev T, Moritz RLV, Moritz RFA (2012) Spatial and temporal dynamics of the male effective population size in bumblebees (Hymenoptera: Apidae). *Population Ecology* **54**, 115–124.

Zayed A, Packer L (2005) Complementary sex determination substantially increases extinction proneness of haplodiploid populations. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 10 742–10 746.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Reproductive strategies of males from three main eusocial Apidae bees.

Figure S2 Trap-nests lodged within the campus of University of São Paulo, Brazil, which received mating swarms of *Tetragonisca angustula* males.

Table S3 Number of males donated by each of the 83 colonies and their distribution in each mating swarm.