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Sociogenetic structure in nests of the mud dauber wasp *Trypoxylon* (*Trypargilum*) *albitarse* (Hymenoptera: Crabronidae)

Antonio C. Bragato Bergamaschi[1], Juliano da Costa Almeida[1], Lucio A. de Oliveira Campos[2] and Marco A. del Lama[1]

[1]Departamento de Genética e Evolução, Universidade Federal de São Carlos, 13565-905 São Carlos, SP, Brazil; e-mails: acbbergamaschi@gmail.com; juliano03@gmail.com; dmdl@ufscar.br

[2]Departamento de Biologia Geral, Universidade Federal de Viçosa, 36570-000 Viçosa, MG, Brazil; e-mail: lcampos@ufv.br

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Abstract. *Trypargilum* is a subgenus of solitary spider-hunting wasps whose males guard the nest, an unusual behaviour for male wasps. A male pairs with a female and copulates repeatedly with her during the nesting process, although females regularly copulate with satellite males, which employ an alternative reproductive strategy. The purpose of this paper was to determine the sociogenetic structure in twenty-nine nests of *Trypoxylon albitarse* sampled at six sites in Brazil. A total of 367 wasps were genotyped for eight species-specific polymorphic microsatellite loci. Genotypic segregation analyses were conducted to test whether the nests sampled were monogamic family groups. The results indicated that all the offspring in 12 of the 29 nests could be attributed to a single couple (genetic monogamy). Approximately 9% of the offspring probably resulted from extra-pair copulations and 3% of the total offspring were attributed to a second mother (usurpation by conspecific females, a form of intraspecific parasitism). The sequential replacement of parents throughout the nesting process indicates that the 29 nests analyzed included 35 family groups. Thus, our findings indicate that *Trypoxylon albitarse* has a predominantly monogamous genetic mating system, despite the social polygamy reported in previous studies.

INTRODUCTION

Trypoxylon is a diverse genus of solitary wasps of the family Crabronidae (Hymenoptera) whose females display parental care during the construction and provisioning of nests in which their offspring develop to the adult stage (Coville, 1982). This genus is divided into two subgenera: *Trypoxylon* and *Trypargilum* (Richards, 1932).

Trypargilum nests are usually guarded by a male during provisioning, which is unusual among Hymenoptera and other insects (Brockmann & Grafen, 1989). This territorial behavior requires *Trypargilum* males to invest considerable time and energy in a single partner, but presumably increases their chances of reproductive success by defending their territory from other males and repeatedly mating with the female during the nesting process (Coville & Coville, 1980). Despite the formation of breeding couples that remain together throughout the nesting process, previous behavioral studies have revealed a complex mating system in species of *Trypargilum,* including alternative reproductive tactics, in which satellite males remain close to active nests and persistently attempt to mate with females already assisted by a different male (Coville & Coville, 1980; Brockmann & Grafen, 1989; Amarante, 1991; Buschini & Donatti, 2012).

Trypoxylon albitarse is widely distributed throughout the Neotropical region (Amarante, 2002). Like other species of the Albitarse Group, females build mud tubes that have a rough appearance due to the way the mud is applied in the form of inverted V-shaped rows (Fig. 1). These nests usually are built on vertical surfaces sheltered from rain with the entrance facing the ground; they are often found on the walls of man-made constructions located near parks and forests (Amarante, 1991). Details of the nesting biology of this species can be found in Brunch (1932), Rau (1933), Fritz & Genise (1980) and Amarante (1991).

Amarante (1991) studied the nesting behaviour of *T. albitarse* and reported repeated copulations between the male guarding the nest and the nesting female throughout the nesting process, especially before oviposition. However, this author also reports the frequent presence of satellite males positioned near the nest entrance awaiting the arrival of the female, when extra-pair copulations were recorded. The receptivity of *T. albitarse* females already accompanied by a male partner to extra-pair copulations raises doubts regarding the effectiveness of the guarding strategy of the resident male and the real significance of this behavioural trait, which is shared by nearly all of the species of *Trypargilum* (Coville, 1982; Brockmann & Grafen, 1989).

The purpose of the present study was to determine the sociogenetic structure of the offspring in nests of *T. albitarse* using eight species-specific polymorphic microsatellite loci and test the hypothesis that the mating system is essentially monogamous.

MATERIAL AND METHODS

Study areas and fieldwork

The fieldwork was conducted between 2005 and 2012. *T. albitarse* nests were collected from six sites in Brazil: (1) the campus of the Federal University of São Carlos (UFSCar) and surrounding areas in the city of São Carlos in the state of São Paulo (SP) (22°01´S, 47°53´W); (2) the UFSCar campus in the city of Ara-

Fig. 1. *Trypoxylon albitarse* female using mud to build a tube in the form of rows of inverted V-shaped cells.

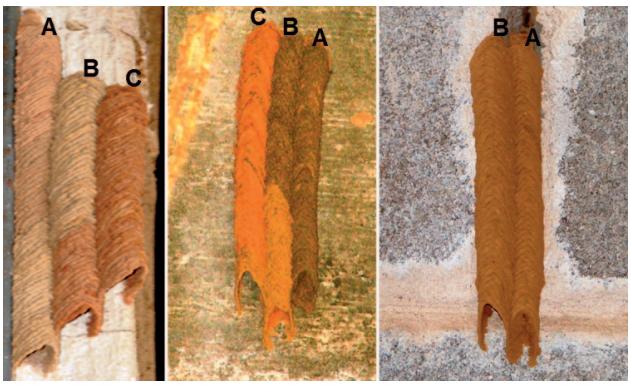

Fig. 2. Examples of the nests of *Trypoxylon albitarse* sampled. The sequence in which the mud tubes were built (represented by letters A, B or C) was inferred based on their juxtaposed position, with a new tube wall constructed using part of the wall of a previously built tube.

ras, SP (22°18′S, 47°22′W); (3) Angelim Farm in the city of Ubatuba, SP (23°23′S, 45°03′W); (4) Santo Antônio Farm in the city of Duartina, SP (22°24′S, 49°24′W); (5) the campus of the Federal University of Viçosa (UFV) in the city of Viçosa in the state of Minas Gerais (MG) (20°47′S, 42°52′W); and (6) the campus of the Santa Cruz State University (UESC) in the city of Ilhéus in the state of Bahia (BA) (14°38′S, 39°06′W).

Trypoxylon albitarse nests found on the walls of man-made buildings were carefully opened with the aid of fine-tipped tweezers. If offspring in the pupal stage were found, the cocoons were collected and placed in individual plastic bags. Each set of parallel tubes was treated as a single nest and a priori considered as a family group. Each specimen sampled was properly identified by the location and number of each nest, tube and cell from which it was removed. The sequence in which the mud tubes were built was inferred based on their juxtaposed positions, with a new tube wall constructed using part of the wall of a previously built tube (Fig. 2). Some nests were observed during fieldwork until they were completed at which time the male or female or both wasps in attendance were collected. The pupae were transported to the laboratory and the offspring was maintained at room temperature until they reached the adult stage, when it was possible to determine their sex. The adult specimens were then stored at –20°C for subsequent DNA extraction.

DNA extraction and microsatellite analysis

Total DNA was extracted by maceration of the mesosoma or legs of adult specimens using the phenol-chloroform (Fernandes-Salomão et al., 2005) or Chelex (Walsh et al., 1991) protocols. Nine species-specific microsatellite loci prospected and characterized by Almeida et al. (2013) were included in the genotyping (Talb01, Talb02, Talb03, Talb05, Talb06, Talb07, Talb09, Talb12, Talb14), but one of them (Talb12) was non-polymorphic and excluded from the analysis. Polymerase chain reactions were performed using 250 µM of each dNTP, 2.5 mM of $MgCl_2$, 0.5 µM of fluorescent labelled forward primer, 0.5 µM of unlabelled reverse primer, 1× BioTools buffer and 1 U of Taq DNA polymerase (BioTools, Madrid, Spain), in a final volume of 10 µL. Amplifications were performed using an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany) and the following procedure: initial denaturation at 94°C for three minutes, followed by 35 cycles of 30 s at 94°C (denaturation), 20 s at the specific hybridization temperature for each amplicon indicated by Almeida et al. (2013) and one minute at 72°C (extension). The reaction was completed with a final extension step at 70°C for 30 min.

Genotyping was carried out after running the amplified DNA fragments in a MegaBace-1000 automated sequencer (GE Healthcare, Buckinghamshire, UK). Allele sizes were obtained by reading the peaks in the software MegaBace Fragment Profiler 1.2 (GE Healthcare), which were then compared with the peaks of the ET550R marker. To increase the reliability of the final dataset, approximately 25% of the specimens were genotyped twice especially those from nests at which more than one father or mother or diploid males were recorded. The high hit rate obtained using this method, the successful amplification of the males sampled and the very low proportion of diploid males made it unnecessary to use correction methods or to detect null alleles, which are usually done in analyses involving microsatellite markers.

Data analysis

An assignment test was performed using the GenAlex 6.5 program (Peakall & Smouse, 2006) to estimate the probability of each specimen being attributed to the original nest and test the reliability of the genetic markers used in the analyses. Like other Hymenoptera, *T. albitarse* is a haplodiploid species, with haploid males and diploid females. As the GenAlex 6.5 program does not distinguish between haploid and diploid individuals in the same database, the population of females was doubled and males were considered homozygous in this analysis to conserve the allelic proportions. Despite underestimating the population of males, the assignment test is based on allele frequencies, making this parameter irrelevant to the results obtained.

The genetic structure of the nests sampled was established by visual inspection of the genotypes of the offspring and, whenever possible, the presumed mother and father. This method was facilitated by the haplodiploid sex-determination system. Under monandry, female offspring of a mother carry the same paternal genotype, allowing the deduction of the paternal allele for each locus. The genotypes of male offspring can be used to reconstruct the maternal genotype. To avoid errors in the reconstruction of parental genotypes, only nests with more than seven genotyped wasps were included in the analysis.

To support pedigree determination based on the visual inspection of genotypes, genetic relationships were determined using the maximum likelihood procedure in the Kingroup 2 program (Konovalov et al., 2004), following the method described by Goodnight & Queller (1989) and Queller & Goodnight (1999). Tests were performed using female genotypes through a series of 100,000 paired simulations based on allele frequencies previously obtained from a reference population. Since the wasps were collected from six different sites and only the São Carlos site had a significant pool of unrelated individuals (37 females obtained from 37 different nests), only the nests sampled from this site

were submitted to this analysis. In the first step, female genotypes from each nest were analyzed separately. Pairwise comparisons allowed the determination of full-sister relationships ($R = 0.75$). For nests in which the nesting female was collected, mother-daughter relationships were also tested ($R = 0.5$).

Using the same software, the probability (P) of pairs of females sharing alleles by descent was analyzed. The hypotheses were defined as null hypothesis (H_0 – full sisters) and alternative hypothesis (H_1 – absence of genetic relatedness). The Kingroup 2 program allows the haplodiploid sex-determination system to be taken into consideration in this analysis, which takes into account that full sisters should share an average of 50% of the genes inherited maternally and 100% of genes inherited paternally. Pairwise comparisons between female offspring were performed for individuals belonging to the same nest as well as between female offspring from different nests. To test the agreement between the two methods employed to determine the genetic structure of *T. albitarse* nests, detailed comparisons were made between the results obtained from the visual inspection of the genotypes and those obtained using the Kingroup 2 program.

RESULTS

Twenty-nine nests of *T. albitarse* with at least seven specimens available for genotyping were used to determine their genetic structure. A total of 367 wasps were genotyped (193 females and 174 males), resulting in mean of approximately 12 individuals per nest. For 22 nests, it was only possible obtain the genotypes of the brood. In addition, the resident male (presumed father) for one nest, the nesting female (supposed mother) for three nests and both parental candidates for three nests (Table 1) were collected for analysis.

The specimens sampled were genotyped for nine species-specific microsatellite loci, which are described and characterized by Almeida et al. (2013). As found by these authors, locus Talb12 also exhibited no genetic polymorphism in the present samples and was therefore excluded from the analysis. Almeida et al. (2013) used a population from São Carlos as reference for the characterization of the loci, which was from the same location of 19 of the nests sampled in the present study (Table 1). The genotype analysis performed by the authors did not suggest deviations from Hardy-Weinberg equilibrium or pairwise linkage disequilibrium between loci, respectively.

The assignment test indicated that 90% of the specimens were correctly assigned to their original nests. An alternative assignment test was also conducted excluding specimens that the genotypic segregation indicated could not be full siblings, resulting in an increase in this proportion to 92% of specimens correctly assigned to their original nests.

Table 2 shows the results obtained for the determination of the genetic structure of the twenty-nine *T. albitarse* nests both by using visual inspection of the segregation of genotypes (some examples can be found in the Supplementary Materials available online) and estimated using the Kingroup2 program. Our findings allowed the characterization of four distinct situations: (1) for 12 nests the brood was attributed to a single couple, revealing genetic monogamy; (2) the presence of more than one father was presumed to explain the genotypes of the daughters in 16 nests; in this case, 21% of the female offspring were considered to be the daughters of a second or third father; (3) considering the position of the cells in those 16 nests, sequential replacement of father was detected in ten nests, possibly indicating that a satellite male assumed the nest guard position and fathered the brood in the last cells in these nests. If we exclude the cases of sequential replacement of males during the nesting process, the proportion of daughters generated by extra-pair copulations decreased from 21.6% to 9.1%; (4) the offspring was assigned to more than one mother in seven nests, representing 7.3% of the total brood. However, considering the positions of the cells and tubes there is evidence of a sequential replacement of the nesting female (a different mother was found at the last cell or cells stored), which decreased the proportion of brood that probably resulted from nest usurpation to 3.1%.

As mentioned in the Materials and Methods, kinship analyses were conducted using the Kingroup 2 program only for the 19 nests sampled in São Carlos. The coefficients of relationship (R) obtained in the pairwise comparisons between females of each nest were carefully compared and they corroborated most of the results obtained using visual analysis of genotypic segregation. This also allowed the determination of mean genetic relatedness (mean R) for the female offspring in each nest analyzed

Table 1. Nest codes, number of male and female offspring and presence of potential parents recorded at the twenty-nine nests of *Trypoxylon albitarse* at six sites in Brazil that were used in the genetic analysis. Location codes: SCL – São Carlos (SP), ARR – Araras (SP), DUA – Duartina (SP), VIC – Viçosa (MG) and ILH – Ilhéus (BA).

Nest Code	Female Offspring	Male Offspring	Nesting Female	Guard Male
SCL13	6	4		
SCL16	5	5		
SCL17	8	2		
SCL33	5	4		
SCL109	3	2	X	X
SCL124	2	4		X
SCL133	9	13	X	X
SCL142	5	7	X	
SCL145	6	5		
SCL148	6	5		
SCL151	6	11		
SCL153	8	10		
SCL154	11	9		
SCL157	5	4		
SCL158	4	11		
SCL159	10	10		
SCL160	4	10		
SCL163	15	8		
SCL166	8	5	X	X
ARR1	3	12		
ARR13	10	5		
ARR18	7	2		
UBA6	6	6		
DUA3	10	1		
VIC2	7	3		
VIC11	4	5		
VIC12	6	0	X	
VIC25	4	4	X	
ILH2	4	3		
TOTAL	187	167	6	4

Table 2. Genetic structure of 29 *Trypoxylon albitarse* nests sampled at six sites in Brazil. Acronyms: NPM – inferred number of potential mothers; NPF – inferred number of potential fathers; EPC – extra-pair copulations based on the proportion of female offspring assigned to another father; BAOM – brood attributed to another mother based on the detection of a usurping female or sequential mother exchange; FS – full-sister; MD – mother-daughter. Location codes: SCL – São Carlos (SP), ARR – Araras (SP), DUA – Duartina (SP), VIC – Viçosa (MG) and ILH – Ilhéus (BA).

NEST CODE	MENDELIAN ANALYSIS				LIKELIHOOD ANALYSIS	
	NPM	NPF	EPC	BAOM	FS relationship Mean *R* (± SE)	MD relationship Mean *R* (± SE)
SCL13	1	1			0.84 (± 0.07)	–
SCL16	1	2	1/5		0.62 (± 0.33)	–
SCL17	2	2	1/8	1/10	0.75 (± 0.23)	–
SCL33	1	2	2/5		0.76 (± 0.16)	–
SCL109	1	2	1/3		0.49 (± 0.27)	0.45 (± 0.07)
SCL124	1	1			0.9	–
SCL133	1	2	3/9		0.61 (± 0.21)	0.66 (± 0.13)
SCL142	1	2	3/5		0.72 (± 0.12)	0.79 (± 0.07)
SCL145	1	1			0.84 (± 0.23)	–
SCL148	1	1			0.72 (± 0.13)	–
SCL151	2	4	1/6, 1/6, 1/6	3/17	0.52 (± 0.21)	–
SCL153	1	2	2/8		0.77 (± 0.12)	–
SCL154	1	1			0.77 (± 0.09)	–
SCL157	2	1		4/9	0.59 (± 0.09)	–
SCL158	1	2	2/4		0.48 (± 0.26)	–
SCL159	1	4	2/10, 1/10, 1/10		0.5 (± 0.08)	–
SCL160	2	3	1/4	1/14	0.67 (± 0.2)	–
SCL163	2	4	2/15, 3/15, 6/15	4/23	0.52 (± 0.26)	–
SCL166	3	2	1/8	4/13, 1/13	0.54 (± 0.21)	0.39 (± 0.15)
ARR1	2	2	1/3	8/13	–	–
ARR13	1	1			–	–
ARR18	1	1			–	–
UBA6	1	1			–	–
DUA3	1	1			–	–
VIC2	1	3	1/7, 2/7		–	–
VIC11	1	1			–	–
VIC12	1	1			–	–
VIC25	1	1			–	–
ILH2	1	3	1/4, 1/4		–	–
TOTAL			21.9%*	7.3%*		

as well as the genetic relatedness between the presumed mother and female offspring for cases in which the nesting female was also sampled (Table 1).

Estimates of the probability of female offspring sharing alleles by descent revealed that only about 7.5% of pairwise comparisons between females from different nests indicated related individuals. Otherwise, nearly 26% of the comparisons between the female offspring of the same nest indicated no genetic relationship. As previously reported for *R* estimates, *P* values obtained between pairs of females from the same nest demonstrated strong agreement with the results obtained from the visual inspection of the genotypic segregation.

The genetic data revealed unexpected situations occurring during the provisioning of the nest: (1) In the case of nest SCL163 wasps were sampled from seven mud tubes. A simple genetic structure was detected in the first five tubes (only one father and one mother explain the genotypes). However, the first and a second female shared the maternity of the brood in the last two tubes with a second father, showing a sequential exchange of the guarding male as well as conspecific parasitism; moreover, this male mated with both partners; (2) Nest SCL157 was composed of three mud tubes. A second mother was responsible for the brood in the last cells in the last two tubes, but a single male fathered all of the female brood; (3) In Nest SCL151, a different female-male pair was responsible for the brood in a tube located between two other tubes, indicating that the construction and provisioning the same nest may take place simultaneously in different tubes by distinct couples.

Five diploid males found in two of the 29 nests analyzed were identified among the 172 males genotyped.

DISCUSSION

In this study, the sociogenetic structure of the offspring in nests of *T. albitarse* was determined using both genotypic segregation and maximum likelihood, with similar results. This is supported by a comparison of the data in Table 2, which indicates a positive association between the mean *R* for female offspring and the number of extra-pair

copulations. In other words, mean *R* values were usually smaller for nests in which the genotypic segregation analysis indicated extra-pair copulations. As expected, mean *R* values were usually lower for nests in which genotypic segregation indicated that the female offspring could be assigned to more than one parent in comparison to the values for nests for which only one mother was detected.

Amarante (1991) recorded *T. albitarse* females mating with satellite males. The social polygamy reported by this author is corroborated by the present genetic analyses, but the low proportion of daughters originating from extra-pair copulations (9%) indicates that the genetic mating system of *T. albitarse* is predominantly monogamous (genetic monogamy) as the guard male fathered the female brood in the four nests whose guarding male was analyzed.

Considering those cases in which the father, mother or both parents are replaced, our results indicate that the 29 nests sampled are actually made up of 35 genetic nests or family groups. The analysis of the genotypic segregation of the offspring in the 29 nests (not excluding cases of sequential replacement of parents) revealed that about 22% of the female offspring could be assigned to a second or third father (Table 2). When the 19 nests sampled at São Carlos were subjected to a likelihood analysis in order to estimate the coefficients of relatedness, nearly 26% of the pairwise comparisons of females from the same nest revealed unrelated individuals. Comparing the results obtained by the two methods, nest by nest, nearly all the specimens considered to be unrelated by genotypic segregation were also unrelated in the Kingroup2 analysis. However, some specimens considered related using the former procedure were determined to be unrelated using the latter procedure, resulting in a slight increase in the proportion of broods assigned to other parents. Thus, the two methods yielded similar results. As mentioned above, the values recorded for the absence of relatedness between individuals from the same nest were due to the sequential replacement of the father, mother or both, in addition to extra-pair mating. So, our findings indicate that nests can be formed by more than one pair.

Behavioural records indicate that females of some species of *Trypargilum* that copulate with another male mate again with the resident partner when they return to the nest (Garcia & Adis, 1995; Buschini & Donatti, 2012; Bergamaschi A.C.B., unpubl.). When a female copulates with more than one male, it can result in sperm competition. Studies indicate that the last partner to mate prior to oviposition is most likely to father the offspring (Parker, 1984). This process is called sperm precedence and is recorded in many species of Hymenoptera (Michener, 2007). It is likely that this has resulted in the evolution of several reproductive strategies, such as: (1) males guarding females after copulation until the time of oviposition; (2) males remaining near the nest; and (3) males staying in active nests and awaiting the return of the female (Alcock, 1978; Thornhill & Alcock, 1983). In all these cases the male spends time and energy to be the last one to mate prior to oviposition, which indicates that this is an effective way of achieving paternity of the brood. Banks (1995) found a positive correlation between the time spent guarding a nest and the number of times males of *Cerceris binodis* (Hymenoptera: Crabronidae) copulate. The same may occur in species of *Trypargilum*.

Trypargilum females mating repeatedly is considered a high cost in terms of energy (Daly, 1978; Thornhill & Alcock, 1983; Lewis Jr, 1987). However, despite this potential cost, their receptivity to repeated copulations may be explained by the findings described by Moreira (2007), who analyzed the morphology of the female spermatheca of species of the subgenera *Trypoxylon* and *Trypargilum*. This author found that this organ is smaller in *Trypargilum* than in *Trypoxylon* s. str. females, which may account for why the former requires copulation prior to each oviposition to produce daughters. Thus, for a better understanding of this complex mating system, studies on the morphology of the reproductive tract of *Trypargilum* are an essential requirement for testing hypotheses about sperm precedence and the functional morphology of spermatheca.

During field sampling, satellite males of *T. albitarse* were frequently observed attempting to occupy nests guarded by a male. Amarante (1991) also reports similar confrontations in this species, in one of which the resident was dislodged by the rival after a fight started inside and finished outside the nest. Similar studies on other species of the subgenus also report fighting between males, which, in some cases, was followed by the displacement and replacement of the resident guard male, see Coville & Coville (1980) for *Trypoxylon tenoctitlan* and Coville et al. (2000) for *Trypoxylon lactitarse* in Costa Rica, Brockmann & Grafen (1989) for *Trypoxylum politum* in the United States, Garcia & Adis (1995) for *Trypoxylon rogenhoferi* and Buschini & Donatti (2012) for *Trypoxylon agamemnon* in Brazil. Thus, intrusion attempts and partner exchanges after a fight seem to be common in *Trypargilum* nests. The genetic findings of the present study corroborate these observations, as the sequential replacement of the father was detected in *T. albitarse* nests, which did not necessarily occur at the beginning of a new mud tube.

In addition to the sequential exchange of the father, our data also revealed nests with more than one mother. According to Brockmann (1980), *Trypargilum* females that nest in mud tubes can adopt different nesting strategies: (1) the use of a nest previously occupied by another female; (2) two females can provision the same nest without even meeting one another and the male can mate with both females; (3) a female intruder can open the wall of the tube and replace the original egg with one of her own (intraspecific cleptoparasitism). We found evidence of these three strategies in the *T. albitarse* nests studied. The data on genetic structure clearly revealed the sequential replacement of females in some nests. In one of the nests monitored during fieldwork, two females were observed simultaneously stocking paralyzed spiders in the same brood cell, which would explain why the offspring of some cells were assigned to another mother (usurper female). We also recorded nests in which the walls had a circular opening cov-

ered with mud of a different colour. Specimens of *T. albitarse* emerged from these cells, but, unfortunately, the low number of wasps sampled from these nests did not allow us to test the genetic origin of these individuals.

There were five diploid males among the 172 males genotyped. These five males came from two different nests. It is noteworthy that two of these diploid males were also characterized as heterozygous for allozyme markers (Almeida J.C., unpubl.), reinforcing their diploid status. The expected ratio for a couple who shares the same allele for the complementary sex determination (csd) locus is one female to one diploid male. This proportion was not recorded for these two nests because more than one mother could account for the genotypes of the brood.

CONCLUSION

The present findings provide the first empirical evidence that despite the social polygamy recorded for *T. albitarse* females, a predominant father was recorded for each nest, indicating that this species has a predominantly monogamous genetic mating system. It is worth noting that we recorded that the guard male was the only or the predominant father for four of the nests analyzed. This suggests that guarding behaviour by *T. albitarse* males probably increases their chances of fathering female offspring. Further studies are required to test the hypothesis that the probability of paternity is increased by male guarding and multiple copulations in other species of the subgenus *Trypargilum*.

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