Genetic analyses reveal female philopatric behavior and nest usage by multiple females of the solitary oil-collecting bee *Tetrapedia diversipes* (Hymenoptera: Apidae)

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Abstract – *Tetrapedia diversipes* is an oil-collecting solitary species widely distributed in the Neotropical region. The high incidence of this species in trap nests makes it potentially a model species for ecological, genetic, evolutionary, and physiological studies. Here, we performed a population genetic study by using mitochondrial and microsatellite markers. This study aimed to investigate the genetic diversity, gene flow among populations, and offspring relatedness within the nest. The results indicated female philopatry and gene flow to be mainly mediated by males. Data on offspring relatedness suggested that nest owner replacement is common in this species.

Atlantic forest / solitary bee / relatedness / kinship / cytochrome C oxidase I (COI) / microsatellites / trap-nest

1. INTRODUCTION

Tetrapedia diversipes Klug (1810) is a solitary oil-collecting bee of the tribe Tetrapediini which was recently coupled with *Ctenoplectra* in the subfamily Xylocopinae (Apidae) (Bossert et al. 2018). *Tetrapedia diversipes* has a wide geographic distribution, ranging from Costa Rica to Argentina (Moure 2012). The female builds the nest in pre-existing holes in tree trunks. Each nest is composed of an average of seven individual and serial cells. Cells are filled with a mass of pollen

mixed with floral oil where the female lays the egg, and the cells are separated from each other by a layer of sand mixed with floral oil (Alves-dos-Santos et al. 2002). After accomplishing the reproductive task, the female dies; therefore, there is no generation overlap (Aguiar and Martins 2002; Alves-dos-Santos 2003; Michener 2007; Gazola and Garófalo 2009). This species presents a high occupancy rate in trap nests which may be related to its gregarious behavior, as when more females occupy one site, the cavities increase in attractiveness to other females (Cordeiro et al. 2019). It is likely that nests may be reused by the offspring of the previous generation born on that site (i.e., philopatry) (Cordeiro et al. 2019); nonetheless, there is still no data for *T. diversipes* that supports this hypothesis. The high incidence of this species in trap nests gives it the potential to be a model organism to perform genetic, physiological,

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ecological, and evolutionary studies (Alves-dos-Santos et al. 2002; Cordeiro et al. 2011, 2019; Menezes et al. 2012; Pinto et al. 2015; Rocha-Filho and Garófalo 2015; Araujo et al. 2016, 2017; Santos et al. 2018). However, despite the studies already performed for the species, the population dynamics, brood relatedness, mating system, and nest occupation are not yet fully understood.

Genetic analysis has been well stated as an efficient approach to answer questions of ecology and genetics. In attempts to understand population dynamics, genetic diversity, relatedness, sex dispersal pattern, species richness, and measure of the anthropogenic impacts, studies using molecular markers allied to population genetic analysis tools have already been conducted on bees (Freitas et al. 2009; Carvalho and Del Lama 2015; dos Santos et al. 2016a, b; Francisco et al. 2016b; Frantini-Silva et al. 2017; López-Urbe et al. 2017; Lozier and Zayed 2017; Chapman et al. 2018; Françoise et al. 2018). We performed genetic analysis using mitochondrial and nuclear molecular markers in order to resolve some of the basic questions concerning *T. diversipes* general biology.

2. MATERIAL AND METHODS

2.1. Samples

Trap nests were set in four Atlantic Rain Forest conservation areas in the state of São Paulo: Estação Biológica de Boracéia (Bora) (23° 38' S/45° 52' W), Parque Estadual da Serra da Cantareira (Cant) (23° 22' S/46° 36' W), Parque Estadual da Ilhabela (Ilha) (23° 45' S/45° 27' W), and Parque das Neblinas (Nebl) (23° 84' S/45° 32' W) (Cordeiro et al. 2019). The nests were collected during March 2007 to February 2008 and kept in the laboratory, and the emerging individuals were deposited at the Coleção Entomológica Paulo Nogueira-Neto (CEPANN), Instituto de Biociências, USP. Additionally, trap nests set at the University of São Paulo (USP), city of São Paulo (23° 33' S/46° 43' W) were sampled between September 2012 and February 2013. This latter population has been well established since 1998. The distance between locations varied from

23 km (Cant to USP) to 144 km (Ilha to USP) (Figure 1).

One female was analyzed per nest, totalling 116 females, with 25, 21, 25, 20, and 25 from Bora, Cant, Ilha, Nebl, and USP, respectively. Detailed information such as species ID, date and collection sites, date of adult's emergence, and the molecular markers assessed can be found in Online Resources 1 - Table S1.

2.2. Molecular data and population analysis

DNA extraction of pinned individuals was performed using the DNeasy Tissue kit (Qiagen, Germany) following the manufacturer recommendations. DNA extraction of the fresh material (individuals from USP) was performed using Chelex protocol according to Walsh et al. 1991 (see Françoise et al. 2016). In both cases, just one middle leg was used as the DNA source.

The mitochondrial gene *cytochrome C oxidase I* (COI) was partially amplified and sequenced using the primers BarbeeF and MtD9 following conditions described in Françoise and Arias (2013). The alignments were performed by the program Muscle (Edgar 2004) included in the Geneious 8.1.9 software (www.geneious.com). DnaSP 5.10.1 (Librado and Rozas 2009) was used to calculate the number of haplotypes and variable sites, haplotype distribution, haplotype diversity (H_d) and nucleotide diversity (π). F_{ST} values (considering conventional F -statistics) and AMOVA and Mantel tests were calculated using Arlequin 3.5 (Excoffier and Lischer 2010). AMOVA was calculated considering all populations as one group using conventional F -statistics and haplotype frequencies. Haplotype network was built using the median-joining algorithm (Bandelt et al. 1999) included in PopART software (Leigh and Bryant 2015).

Ten species-specific polymorphic microsatellite loci were screened (Arias et al. 2016). Amplification and visualization followed Francisco et al. (2011). GenALEX 6.5 (Peakall and Smouse 2006) was used to calculate the allele number and expected and observed heterozygosity (H_e and H_o , respectively); FSTAT 2.9.3 (Goudet 1995), to calculate allelic richness and inbreeding coefficient (F_{IS}) per loci; Arlequin, to calculate the significant value of F_{IS} per population and to estimate F_{ST} and R_{ST}

Figure 1. Collecting sites of *Tetrapedia diversipes* in the state of São Paulo, Brazil. 1, Parque Estadual da Serra da Cantareira; 2, Estação Biológica de Boracéia; 3, Parque Estadual da Ilhabela; 4, Universidade de São Paulo; and 5, Parque das Neblinas. This map was made using QGIS 3.4.7 software (QGIS 2019) using the Coverage and Land Use layer (IBGE 2016).

(this metric takes into account the number of repeats between alleles) (Holsinger and Weir 2009); MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004), to verify homozygote excess for each locus; and GENEPOP 4.2, to test for Hardy-Weinberg equilibrium (HWE). STRUCTURE 2.3.4 software (Pritchard et al. 2000) (MCMC burn-in of 100,000 and 1,000,000 post burn-in iterations) was used to estimate the most probable population number. Twenty independent runs for each K value (1–10) were performed, under two conditions: considering the population origin and not considering the population origin. Individuals were modeled as admixed, and correlations were allowed among allele frequencies (Falush et al. 2003). The results of the independent runs were aligned by using CLUMPP (Jakobsson and Rosenberg 2007), and the determination of delta K by using Structure Harvester (Evanno et al. 2005; Earl and VonHoldt 2012). DISTRUCT was used for graphical visualization (Rosenberg 2004).

2.3. Offspring relatedness analysis

A total of 12 nests from Ilha and their respective offspring (38 adults) were analyzed (Online Resources 1 - Table S2). Ten polymorphic microsatellite loci were screened to infer the probability of sibship through a full-pedigree likelihood method using COLONY-2.0.6.5 (Jones and Wang 2010). To find the probability of individuals being or not siblings, it is necessary to input the program with the species mating system information. As the mating system (monogamous/polygamous) of *T. diversipes* is still an open question (Alves-dos-Santos et al. 2002; Cappellari et al. 2012), all the possible combinations were tested. The result is the probability of each emerging individual being the offspring of the parents assigned. The mating systems with the best probability values for the majority of the individuals were then considered.

3. RESULTS

3.1. Population analysis: mitochondrial marker

A *COI* sequence of 415 bp was obtained for 112 individuals. A total of 18 haplotypes were verified. The mean *Hd* was 0.787, varying from 0.743 (Cant) to 0.227 (USP). Nucleotide diversity was very low between haplotypes, showing just one or two nucleotide substitutions, and the mean was 0.00572 (Table I). Only three haplotypes (H_06, H_16, and H_18) were shared among populations. Hap_16 was the most frequent and present in all populations but USP. The vast majority of haplotypes were only found in one of the sampled populations. The highest number of exclusive haplotypes was observed in Bora (five), and the lowest in USP and Ilha (two). Furthermore, these later populations presented the lowest total haplotype number (three). The haplotype network recognized two distinct groups, one encompassing Bora, Cant, Ilha, and Nebl (BCIN group) and the other Cant and USP (CU group) (Figure 2).

The F_{ST} values were high in all pairwise comparisons. The lowest was verified between Cant and Nebl ($F_{ST}=0.19$) and the highest between USP and Bora ($F_{ST}=0.67$). These data suggest low or nonexistent gene flow mediated by females (Online Resource - Table S3). Indeed, the AMOVA analysis confirmed higher haplotypic variability among populations (90.79%) rather than within populations (9.21%). Although the

Mantel test showed a positive correlation between F_{ST} values and geographical distances, the P value was not significant (correlation coefficient = 0.36; $P > 0.1$).

3.2. Population analysis: microsatellite markers

Ten polymorphic microsatellite loci were used to genotype 116 females from the five populations. The allele number per locus ranged from 3 to 18. The mean allele number varied from 6.8 (Nebl) to 8.6 (Bora), and the mean allelic richness varied from 6.8 (Nebl) to 8.2 (Cant). The mean expected and observed heterozygosity per population varied from 0.715 to 0.759 and 0.643 to 0.695, respectively. The F_{IS} mean values ranged from 0.119 (USP) to 0.054 (Nebl) (Online Resources 1 - Table S4). These values were significant in three populations: Bora, Cant, and USP ($P < 0.05$), indicating inbreeding. Most loci were in HWE, and excess of homozygotes was verified in all populations for specific locus (three loci in Cant and Ilha, two loci in Bora and USP, and one locus in Nebl) (Online Resources 1 - Table S4). The locus T4 presented an excess of homozygotes in all populations and therefore was excluded from the structuring analysis.

The F_{ST} values based on microsatellite markers were low, ranging from 0.018 (Nebl and USP) to 0.045 (Nebl and Ilha) ($P < 0.05$) (Online Resources 1 - Table S5); therefore, genetic structuring was not detected. However, R_{ST} values

Table I. Genetic diversity indices for each population obtained from 415 bp of *cytochrome C oxidase I (COI)* marker. N, number of samples; *h*, number of haplotypes; *he*, number of exclusive haplotypes; *Hd*, haplotype diversity; *k*, average number of differences; π , nucleotide diversity

Sites sampled	N	<i>h</i>	<i>he</i>	<i>Hd</i>	<i>k</i>	π
Bora	25	6	5	0.427	0.773	0.00189
Cant	19	6	3	0.743	2.398	0.00586
Ilha	23	3	2	0.593	1.352	0.00331
Nebl	20	5	3	0.716	0.989	0.00242
USP	25	3	2	0.227	0.233	0.00057
Total	112	18	15			
Average				0.787	2.338	0.00572

indicated moderate differentiation of Ilha in relation to all other populations (R_{ST} values ranging from 0.103 between Cant and Ilha to 0.134 between Ilha and USP, $P < 0.009$) (Online Resources 1 - Table S5). The Bayesian analysis performed by STRUCTURE suggested $k = 4$ (number of subgroups), and no genetic differentiation among populations when individual population origin was not considered. Nonetheless, when the population origin of each individual was considered, the number of subgroups was $k = 2$ and Ilha population was clearly differentiated, such as the R_{ST} results (Figures 3 and 4).

3.3. Offspring relatedness and mating system

We tested all crossing conditions between males and females regarding their being monogamous or polygamous (Online Resources 1 - Tables S6 to S9). The mating systems female/male monogamous and female monogamous/male polygamous presented the highest probability values for the sibship inferences. For the 38 individuals tested, the mating system female/male monogamous resulted in the highest probability values of individuals being the offspring of the

Figure 2. *Tetrapedia diversipes* haplotype network inferred through the *cytochrome C oxidase I* (*COI*) sequence 415 bp long. Dashes on the line correspond to mutation number. Bora, Estação Biológica de Boracéia; Cant, Parque Estadual da Serra da Cantareira; Ilha, Parque Estadual da Ilhabela; Nebl, Parque das Neblinas; USP, Universidade de São Paulo.

Figure 3. The four clusters identified by Structure software based on the microsatellites data (different colors represent each cluster), without considering the origin of the populations. The different locations are as follows: USP, Universidade de São Paulo; Bora, Estação Biológica de Boracéia; Cant, Parque Estadual da Serra da Cantareira; Nebl, Parque das Neblinas; and Ilha, Parque Estadual da Ilhabela.

assigned parents (ranging from 0.47 to 1.0) (Online Resources 1 - Table S6). Under this model, four nests had all individuals as full siblings, whereas in the remaining eight nests, it was verified that at least one individual could be assigned to a different mother (Table II). Under female monogamous/male polygamous model, it was predicted that nine of the twelve nests would contain offspring from more than one female (Table II). Both systems, in some nests considered as founded by more than one female, the offspring emerged on different dates (in a range of 1 to 12 days), while in other nests, all individuals emerged on the same day (Online Resources 1 - Tables S2, S6, and S8), reinforcing that the offspring was from the same reproductive season. It is worth mentioning that independently of the mating system tested, we always verified unrelated individuals in the same nest.

4. DISCUSSION

The F_{ST} values obtained from the mitochondrial data were high in all pairwise comparisons, suggesting population structuring (Wright 1978).

This pattern provides evidence for limited dispersal by females, since the mitochondrial DNA is maternally inherited and thus a good molecular track for female dispersal behavior. Although we cannot discard sampling errors, the presence of several exclusive haplotypes in each collecting site is also an indication of very low current female migration and suggests that females may exhibit philopatry (Rowe et al. 2017). This same scenario and biological interpretation have been commonly reported for several eusocial bee species (Yanega 1990; Francisco and Arias 2009; Brito and Arias 2010; Bonatti et al. 2014; Francisco et al. 2016b). Primitively eusocial and solitary bees are generally not philopatric (Kim et al. 2008; Francisco et al. 2016a; Rosa et al. 2016; Luna-Lucena et al. 2017; Toledo et al. 2017), but some exceptions have been reported (Ferreira et al. 2013; López-Urbe et al. 2014, 2015; Penha et al. 2015). Moreover, the gene flow between the two distinct groups (BCIN and CU) recognized in the haplotype network may be affected by Serra do Mar, a mountain range across the Atlantic rainforest in the coastal areas from Southeastern towards Southern Brazil (Figures 1

Figure 4. The two clusters identified by Structure software based on the microsatellites data (different colors represent each cluster), considering the origin of the populations. The different locations are as follows: USP, Universidade de São Paulo; Bora, Estação Biológica de Boracéia; Cant, Parque Estadual da Serra da Cantareira; Nebl, Parque das Neblinas; and Ilha, Parque Estadual da Ilhabela.

Table II. Number of nests founded by one or multiple females depending on the mating system tested, indicating that independent of the mating system, the nests can be founded by more than one female. In total, 12 nests and their respective offspring were analyzed by using the Colony software

Mate system tested	Nests founded by more than one female	Nests founded by one female
Female and male monogamous	8	4
Female and male polygamous	5	7
Female monogamous and male polygamous	9	3
Female polygamous and male monogamous	3	9

and 2). Although we provide evidence that female dispersal of *T. diversipes* seems to be limited, the presence of the most frequent haplotype H_16 in all populations, except USP, raises the hypothesis that H_16 should be the most ancient in the sampling, and a historical relictual haplotype.

The microsatellite (Mendelian inheritance) data indicated the absence of genetic structuring. This result, coupled with the mitochondrial information, suggests that males likely mediate the gene flow among the populations sampled. The same scenario has been reported for other bees, independent of their social organization (Danforth et al. 2003; Exeler et al. 2008; Cerântola et al. 2011; Francisco et al. 2016b; Chapman et al. 2018), and strongly suggests the importance of males as the dispersing sex and ultimately enabling gene flow. It is worth noting that the R_{ST} analysis showed a moderate differentiation only between the populations from the mainland and the island (Ilha), which was also corroborated by structure results when considering the origin of the individuals. It is plausible that the ocean or other physical features of this environment prevent male dispersal. The sea as a physical barrier for gene flow has also been reported for *Osmia rufa* (Neumann and Seidelmann 2006) and *Euglossa cordata* (Boff et al. 2014), suggesting that male flights from mainland to the island may occur rarely or even be inexistent, on this route.

4.1. Offspring relatedness

The relatedness analysis clearly identified unrelated individuals emerging from the same nest. This strongly suggests that more than one female may have laid eggs in a nest. Previous studies based on in situ observation have suggested that just one female is the founder of each nest. This conclusion was based on the behavior of the resident female evicting those searching for nesting places (Alves-dos-Santos et al. 2002). The authors observed nest “invasion” only in case of founder female death (Alves-dos-Santos et al. 2002).

The behavior of abandoning the previous nest and foundation or usurpation of others during the season is commonly reported for bees and wasps (Černá et al. 2013). We suggest that *T. diversipes* females, as observed for other solitary bees (Jang et al. 1996; Vinson and Frankie 2000; Černá et al. 2013), initially search for pre-existent cavities and then begin provisioning, subsequently laying the egg. If the female lives long enough, she can either fill all the available space with brood cells and close it, or may abandon it, incomplete. This behavior could explain our results, since we verified nests containing offspring of a single female and others containing the offspring of multiple females. The multiple nest usage behavior may have been selected in response to unstable environmental conditions or due to high parasitic pressures, the “risk-spreading” strategy (Černá et al. 2013).

The assignment of offspring to sibling groups indicated that *T. diversipes* females are monogamous, but males may present monogamous or polygamous behavior. The majority of females of bee species are monogamous. The greatest exception is the honey bees which mate with multiple males (Strassmann 2001; Paxton 2005). Bee males are usually described as polygamous, with the exception of honeybees and stingless bees. In general, there is little information about bee mating systems, particularly for solitary and primitively social bees, as mating behavior is rarely observed (Paxton 2005). Genetic analysis of *T. diversipes* spermathecal content is needed to confirm the female mating behavior (Paxton 2005).

5. CONCLUSIONS

The absence of genetic structuring verified by microsatellites, especially among mainland populations, supports asymmetrical sex dispersal, as the males are responsible for gene flow. Conversely, the high values of F_{ST} obtained for mitochondrial marker suggest low female dispersal and philopatric behavior. Our data suggests that *T. diversipes* females have a monogamous mating system but males can be polygamous. Nest abandoning and usurpation are common; thus, emergent adults from a nest may be descendants of more than one female. The present study contributed to the addition of new information on the species population dynamics, mating, and nesting behaviors. Nonetheless, *T. diversipes* has great potential as a biological model of solitary bee in the Neotropics and studies to complete this dataset should be continued.

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AUTHORS' CONTRIBUTION

PKFS, EF, MCA, and IAS conceived the research and experiments; GC collected the samples; PKFS and EF performed the experiments and analysis. PKFS, EF, and MCA wrote the paper. All authors reviewed the final version.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.

Des analyses génétiques révèlent le comportement philopatric des femelles et l'utilisation du nid par plusieurs femelles de l'abeille solitaire récolteuse d'huile *Tetrapedia diversipes* (Hymenoptera : Apidae).

Forêt atlantique / abeille solitaire / parenté / parenté / Cytochrome C oxydase I (COI) / microsatellites / nid-piège.

Genetische Analysen geben Hinweise auf philopatrisches Verhalten und Nestnutzung bei Weibchen der solitären, ölsammelnden Biene *Tetrapedia diversipes* (Hymenoptera: Apidae).

Atlantischer Regenwald / solitäre Biene / Verwandtschaft / Verwandtschaftsgrad / Cytochrome C Oxidase I (COI) / Mikrosatelliten / Nistfallen.

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