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► **To cite this version:**

Flávio Francisco, Leandro Santiago, Rute Brito, Benjamin Oldroyd, Maria Arias. Hybridization and asymmetric introgression between *Tetragonisca angustula* and *Tetragonisca fiebrigi*. *Apidologie*, 2013, 45 (1), pp.1-9. 10.1007/s13592-013-0224-7 . hal-01234701

HAL Id: hal-01234701

<https://hal.science/hal-01234701>

Submitted on 27 Nov 2015

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Hybridization and asymmetric introgression between *Tetragonisca angustula* and *Tetragonisca fiebrigi*

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Received 22 January 2013 – Revised 12 June 2013 – Accepted 4 July 2013

Abstract – The broadly distributed Neotropical stingless bee *Tetragonisca angustula* was once regarded as having two subspecies, *T. angustula angustula* and *T. angustula fiebrigi*. In a recent taxonomic revision, these subspecies were elevated to species status (*T. angustula* and *Tetragonisca fiebrigi*) based on morphology and distribution. While molecular studies show two well-delineated subgroups within the *Tetragonisca*, they are inconclusive as to whether there is gene flow between *T. angustula* and *T. fiebrigi*. We characterize 1,003 specimens from southern and southeastern Brazil using mitochondrial DNA and microsatellite analysis and demonstrate that there is ongoing hybridization and introgression between *T. angustula* and *T. fiebrigi* and suggest that they may be better regarded as subspecies.

species / subspecies / mitochondrial DNA / microsatellites / Meliponini

1. INTRODUCTION

The stingless bees are a broadly distributed and highly speciose group of corbiculate bees (Michener 2007). Colony reproduction occurs when an established colony starts to provision a new nest site with food stores and workers, and eventually a new virgin queen. Mother and daughter nests may

share resources for several months (Nogueira-Neto 1954), restricting dispersal to the flight range of workers, which may be as little as a few meters (van Veen and Sommeijer 2000). This form of reproduction is conducive to the formation of isolated breeding populations and rapid speciation due to limited gene flow between demes (Brito and Arias 2010; Francisco and Arias 2010; Quezada-Euán et al. 2012). However, hybridizations often occur between incipient species, further complicating the taxonomy of the stingless bees as a whole (Franck et al. 2004).

The taxonomy of the Neotropical stingless bee *Tetragonisca angustula* (Latreille, 1811) is particularly complex. Schwarz (1938) suggested that there are two subspecies: *T. angustula fiebrigi* and *T. angustula angustula* basing this diagnosis on (1)

Electronic supplementary material The online version of this article (doi:10.1007/s13592-013-0224-7) contains supplementary material, which is available to authorized users.

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Manuscript editor: Marina Meixner

mesepisternum color (black in *T. a. angustula* and ferruginous in *T. a. fiebrigi*); (2) color of the propodeum side (black in *T. a. angustula* and ferruginous in *T. a. fiebrigi*); (3) abdominal color (darker in *T. a. fiebrigi*). Schwarz also noted that his proposed subspecies had distinct geographic distributions, with *T. a. angustula* occurring from Central America to southern Brazil and *T. a. fiebrigi* being restricted to Argentina (Misiones), Paraguay, and southwestern Brazil (Mato Grosso to Rio Grande do Sul).

Camargo and Pedro (2008) elevated *T. angustula* and *Tetragonisca fiebrigi* to species status based on unpublished data about the morphology of the male genitalia and the presumed reproductive isolation. Barth et al. (2011) corroborated this claim by showing the presence of B chromosomes in *T. fiebrigi* but not in *T. angustula*. Furthermore, Stuchi et al. (2012) showed that *T. fiebrigi* and *T. angustula* have species-characteristic esterase isozyme electrophoretic profiles. However, both studies were based on small sample sizes that may not have encompassed the complete range of phenotypic variability. For example, in another stingless bee species, *Partamona helleri*, there is variation in B chromosome number between colonies and populations (Brito et al. 1997; Tosta et al. 2004).

T. angustula and *T. fiebrigi* have been reported at the same sites in Brazil (Castanheira and Contel 1995; Baitala et al. 2006; Koling and Moretto 2010), indicating that they occasionally occur in sympatry. Additionally, at sites where the species are sympatric, workers (sometimes from the same nest) show heterogenous coloration of the mesepisternum, varying from ferruginous to black, or showing both colors simultaneously (Castanheira and Contel 1995, 2005; Koling and Moretto 2010). Castanheira and Contel (1995) regarded heterogenous coloration as evidence of hybridization between the two (sub)species. Molecular analyses based on isoenzymes (Castanheira and Contel 1995, 2005), RAPDs (Oliveira et al. 2004), and mitochondrial DNA (mtDNA) restriction sites (Koling and Moretto 2010) showed two well-delineated subgroups, but they are inconclusive in resolving the taxonomic

status of *T. angustula* and *T. fiebrigi*. For this reason, we will hereafter use the terms “Angustula” and “Fiebrigi” to refer to these taxa.

To resolve the taxonomic uncertainty surrounding the *Tetragonisca* group, we collected Angustula and Fiebrigi bees from southern and southeastern Brazil without making any subspecies/species assignment based on morphology a priori. We then performed molecular analysis via mtDNA sequencing and microsatellite genotyping to test the alternative hypotheses that there is a single population versus two separately breeding populations.

2. MATERIALS AND METHODS

2.1. Sampling and DNA extraction

We collected 1,003 bees from 456 sites in Santa Catarina, Paraná, São Paulo, Rio de Janeiro, and Minas Gerais states, Brazil (Figure 1). Bees were sampled from nests ($n=126$, one per nest) and on flowers ($n=877$), preserved in 96 % ethanol and transported to the laboratory. The specimens were dried at room temperature for 20 min right before DNA extraction. The thorax of each bee was used for DNA extraction using Chelex[®] 100 (Bio-Rad) according to the protocol described by Walsh et al. (1991).

2.2. Mitochondrial DNA

Primers mtD06 and mtD09 (Simon et al. 1994) were used to amplify a region of cytochrome c oxidase subunit 1 (*COI*), and mtD26 (Simon et al. 1994) and AMB16 (Arias et al. 2008) for cytochrome b (*Cytb*). PCR reactions were set up in a final volume of 20 μ L with 2 μ L of DNA, 1 \times PCR buffer, 3 mM of MgCl₂, 0.4 μ M of each primer, 200 μ M of each dNTP, 1 M of Betaine (USB), and 1 U of *Taq* DNA polymerase (Invitrogen). The amplification conditions consisted of an initial denaturation at 94 °C/5 min, followed by 35 cycles of denaturation at 94 °C/60 s, annealing at 42 °C/80 s and elongation at 64 °C/120 s. An extra elongation step at 64 °C/10 min was performed.

An aliquot (2 μ L) of PCR product was subjected to electrophoresis on 0.8 % agarose gel stained with

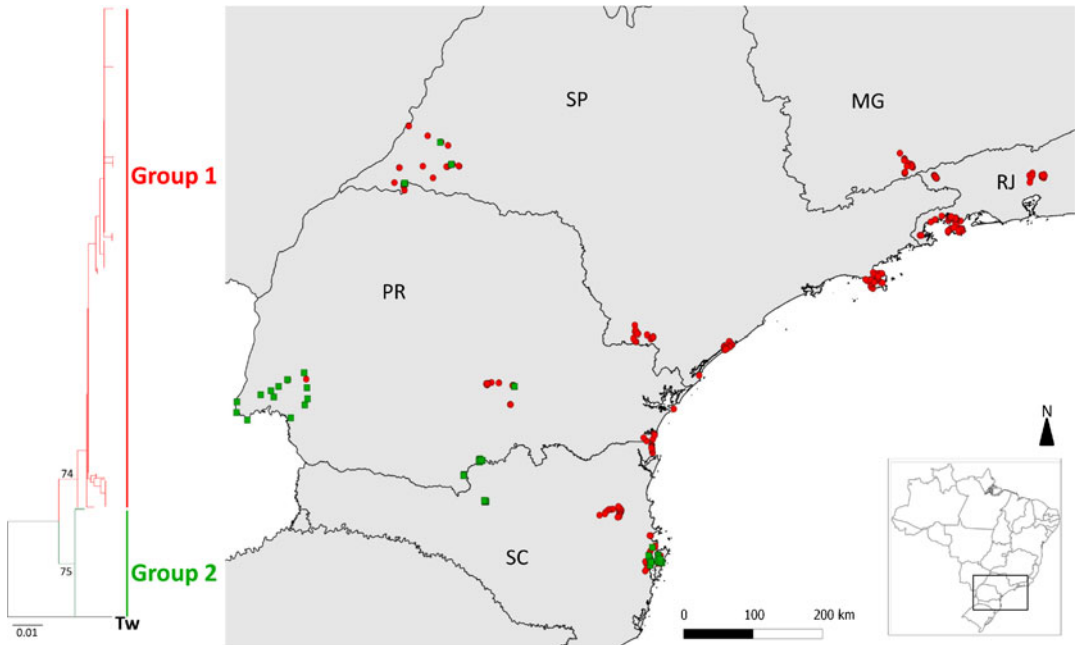


Figure 1. Neighbor-joining tree (left) for *Tetragonisca* samples based on *COI* and *Cytb* sequences. Bootstraps values greater than 70 % are shown. *Tw*: *Tetragonisca weyrauchi*. Map of Brazil (right) showing in detail the geographic distribution of the samples colored according to the two main groups (left).

GelRed™ (Biotium) and visualized under UV light. Positive amplicons (18 µL) were purified with 0.5 µL of ExoSAP-IT™ (USB) and submitted to sequencing (Macrogen, South Korea) using the primers mtD09 and AMB16 for *COI* and *Cytb*, respectively.

DNA sequences were visualized, aligned, edited, and concatenated with the program Geneious 5.1.6 (Drummond et al. 2010). Alignments were performed by “muscle” algorithm (Edgar 2004) with a maximum of eight iterations. A neighbor-joining (NJ) tree with 1,000 bootstrap replications was built by the program MEGA 5.05 (Tamura et al. 2011).

The program DnaSP 5.10.01 (Librado and Rozas 2009) was used to obtain the number of haplotypes and the number of synonymous (dS) and non-synonymous (dN) substitutions. After NJ analysis (see “Results”) we selected one sequence from each of the two major groups found to estimate dS and dN relative to *Tetragonisca weyrauchi* an unequivocal outgroup species that is found

in Peru, Bolivia, and Brazil (Mato Grosso, Rondônia, and Acre) (Camargo and Pedro 2008), but not in the regions that we sampled.

2.3. Microsatellites

PCR components, electrophoresis, visualization, and genotyping were performed according to Francisco et al. (2011). Eleven microsatellite loci were used: Tang03, Tang11, Tang12, Tang17, Tang29, Tang57, Tang60, Tang65, Tang68, Tang70, and Tang77 (Brito et al. 2009). PCR reaction conditions for each locus are presented in Table I.

We used the program Structure 2.3.3 (Pritchard et al. 2000) to estimate the number of subpopulations in our collection. We assumed correlated allele frequencies (Falush et al. 2003). The program was set to 10⁶ iterations after an initial burn-in of 10⁵ iterations. To estimate the number of structured subpopulations (*K*),

Table I. PCR reaction conditions for the 11 microsatellite loci analyzed.

Locus	PCR reaction condition
Tang12 Tang70	96 °C/8 min, 35× (94 °C/30 s, 53 °C/60 s, 72 °C/60 s), 72 °C/10 min, 4 °C/∞
Tang03 Tang57	95 °C/7 min, 6× (94 °C/30 s, 59 °C/30 s, 72 °C/30 s), 6× (94 °C/30 s, 58,5 °C/30 s, 72 °C/30 s), 6× (94 °C/30 s, 57 °C/30 s, 72 °C/30 s), 6× (94 °C/30 s, 56 °C/30 s, 72 °C/30 s), 6× (94 °C/30 s, 55,5 °C/30 s, 72 °C/30 s), 6× (94 °C/30 s, 55 °C/30 s, 72 °C/30 s), 72 °C/5 min, 4 °C/∞
Tang 11 Tang 17	96 °C/8 min, 35× (94 °C/30 s, 60 °C/60 s, 72 °C/60 s), 72 °C/10 min, 4 °C/∞
Tang 29	
Tang 60	
Tang 65	
Tang 68	
Tang 77	

runs were repeated 10 times. We ran $K=2$ because it tests if individuals will be assigned to one or two clusters depending of their membership coefficients. The program Clumpp 1.1.2 (Jakobsson and Rosenberg 2007) was used to align the 10 repetitions. The program Distruct 1.1 (Rosenberg 2004) was used to graphically display the results produced by Clumpp. Microsatellite data were also utilized by Genalex 6.5 (Peakall and Smouse 2006, 2012) to perform a principal coordinate analysis.

3. RESULTS

3.1. MtDNA data reveal two main clusters

Concatenated mtDNA sequences (732 bp) were obtained from all 1,003 individuals and *T. weyrauchi* (GenBank accession numbers KF222891-KF224898) and revealed 79 unique haplotypes. A tree generated from a genetic distance matrix revealed two main subgroups with high bootstrap support (Figure 1). Group 1 consists of 826 individuals. They are distributed mainly near the coast. Group 2 (177 bees) encompassed southwestern sites, although exceptions were observed. In some cases, haplotypes belonging

to the two groups were found in close proximity (<1 km).

3.2. Synonymous substitutions prevail between groups 1 and 2

Sequence comparison analysis showed low divergence between groups 1 and 2 (Figure 1). Most differences (65 %) were synonymous (Table II). Groups 1 and 2 are both well separated from *T. weyrauchi* (Figure 1), with non-synonymous substitutions approximately as frequent as synonymous ones for both groups 1 (51 %) and 2 (53 %) (Table II).

Table II. Number of synonymous (dS), non-synonymous (dN), and total substitutions (dT) observed from sequence comparison between the groups obtained from neighbor-joining analysis (groups 1 and 2; Figure 1), and *Tetragonisca weyrauchi*.

Comparisons	dS	dN	dT
Group 1×Group 2	13	7	20
Group 1× <i>T. weyrauchi</i>	34	36	70
Group 2× <i>T. weyrauchi</i>	30	34	64

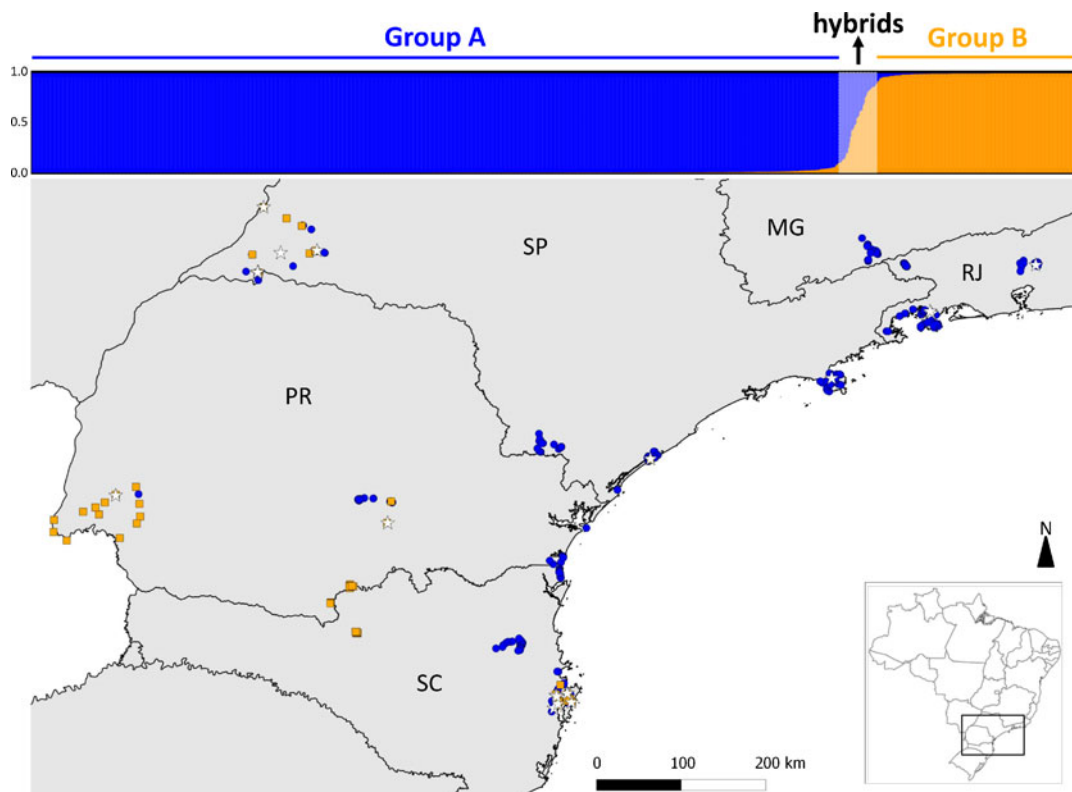


Figure 2. Map of Brazil showing in detail the geographic distribution of *Tetragonisca* individuals from two main groups and hybrids (stars) detected by Structure analysis of microsatellite data (upper panel).

3.3. Microsatellites analysis detects two clusters and asymmetrical hybridization

Analysis based on structure separated bees into two main groups (Figure 2). Hybrids (32

individuals) presenting all proportions of admixture were also detected. The distribution of genotypes was generally congruent with the two groups determined from mtDNA data. Most of the bees from mtDNA Group 1 (773 out of 826) were also in microsatellite group A. Similarly, 164 out of 177 bees from mtDNA group 2 were also in microsatellite group B. Thus, the two main clusters identified by mtDNA and microsatellites were mostly, but not always concordant (Table III). Bees with mtDNA group 1 and microsatellites group B were more frequent and more widespread (15 collection sites in São Paulo, Paraná, and Santa Catarina states) than the opposite (2-A, only five bees from two collection sites in Paraná state). Principal coordinates analysis showed similar results (Figure S1).

Table III. Number of *Tetragonisca* individuals belonging to the groups detected by mitochondrial DNA and microsatellites.

Microsatellite group	Mitochondrial group	
	1	2
A	773	5
B	29	164
Hybrids	24	8

4. DISCUSSION

We suggest that pure *Angustula* individuals belong to mtDNA group 1 and microsatellites group A and pure *Fiebrigi* belong to group 2/ group B. Our data strongly suggest that hybridization occurs between *Angustula* and *Fiebrigi*, and that attempts to distinguish these two taxa based on a single molecular marker are not reliable. The varying degrees of admixture in hybrids suggests that backcrossed and F_2 colonies are viable as also observed in *Tetragonula* (Brito et al. unpublished data). Subspecies are classically defined by their unique phenotypes and geographic distribution, and ability to produce fertile offspring (Mayr 1963). Based on these criteria, we would argue that *Angustula* and *Fiebrigi* are valid subspecies because of the typical separation of mesepisternum coloration, broadly divergent geographical distributions, the rarity of hybrids, and limited gene flow between the two taxa. However we also suggest that they should not be regarded as separate species because of the putative hybrids. In addition, our mtDNA analysis reveals that synonymous substitutions are more frequent than non-synonymous ($dS > dN$) indicating that *Angustula* and *Fiebrigi* are only recently diverged.

Data from both microsatellites and mtDNA sequencing indicated asymmetrical hybridization. Fertilization of *Angustula* queens (AQ) by *Fiebrigi* males (FM) was more frequent than the opposite mating. Asymmetrical introgression has been well documented in the Africanization process of *Apis mellifera* in the Americas (Hall 1990; Rinderer et al. 1991; Lobo 1995; Clarke et al. 2002; Quezada-Euán et al. 2003; Kraus et al. 2007). During this process, the first hybridizations occurred via “African” males mating with “European” queens (Clarke et al. 2002). This asymmetry probably arose because the invading African swarms produced large numbers of males that mated with the resident European queens. AQ×FM crosses were detected at sites where *Angustula* was the predominant taxon, possibly suggesting that *Fiebrigi* colonies produced larger numbers of males. A

further possibility is that differences in male genitalia or mating behavior may favor matings in this direction. Asymmetrical hybridization arising from differences in mating behavior is known from tree frogs (Lamb and Avise 1986), fishes (Lajbner et al. 2009) and leafhoppers (Heady et al. 1989).

According to the theory of adaptive dynamics, a monomorphic population can achieve a phenotypic state wherein ecological interactions induce different selective pressures, leading to a split into two coexisting phenotypic groups (Dieckmann and Law 1996; Dieckmann 1997; Geritz et al. 1998; Dieckmann and Doebeli 1999). Speciation in these coexisting groups can then occur via the accumulation of genetic differences between the incipient species (Coyne 1992; Wu and Palopoli 1994; May-Itzá et al. 2009, 2012; Quezada-Euán et al. 2012). Reciprocal monophyly in mtDNA sequences and high divergence at nuclear loci is evidence of well-differentiated sister species (Moritz 1994). In our study, we observed reciprocal monophyly from mtDNA data but evidence of hybridization with the microsatellite data. This suggests that the time elapsed since *Angustula* and *Fiebrigi* diverged is insufficient to prevent gene flow. Therefore, there is strong evidence that *Angustula* and *Fiebrigi* are not fully established species.

The secondary contact between *Angustula* and *Fiebrigi* may be a consequence of deforestation. *Tetragonisca* is flexible in its requirements for food and nesting sites (Cortopassi-Laurino et al. 2006; Michener 2007). Nests can be found in hollow trees, wall crevices, PET bottles, dry calabashes, and water pipes. This lack of fastidiousness enables *Tetragonisca* to colonize urban areas, and may have brought *Angustula* and *Fiebrigi* into recent contact. This process has been called “hybridization of the habitat” (Anderson 1948) and has been reported for plants, birds, fish, and amphibians (Rhymer and Simberloff 1996). If new genetic combinations are beneficial or neutral for the hybrids, there will be a trend to homogenize the gene pool of the whole species complex (Seehausen et al. 2008).

Angustula and *Fiebrigi* are commonly cultivated in Latin America for commercial purposes or hobby

(Nogueira-Neto 1997; Cortopassi-Laurino et al. 2006). It is favored because of their wide geographical distribution (Camargo and Pedro 2008), docile behavior, and high quality honey (Nogueira-Neto 1997). Nest transportation and trading is very common among beekeepers. We found anecdotal evidence that beekeepers from the Santa Catarina coast (the natural range of *Angustula*) often transport nests from the west of the state (the natural range of *Fiebrigi*). Nest transport mediated by beekeepers may be contributing to end of the allopatry originally described for *Angustula* and *Fiebrigi*. Beekeeping activities has been already suggested as major contributor to introgression and hybridization in *Tetragonula* (Brito et al. unpubl. data), *Melipona* (Nascimento et al. 2000), and *A. mellifera* (De la Rúa et al. 2009).

In conclusion, if we apply the Biological Species Concept (Mayr 1942; de Queiroz 2005), *T. angustula* and *T. fiebrigi* should not be considered separate species but subspecies. A comprehensive phylogenetic analysis of the *Tetragonisca* including *Angustula* and *Fiebrigi*, *T. weyrauchi*, and *T. buchwaldi* (Camargo and Pedro 2008) would shed further light on the evolutionary antecedents of this incipient, but perhaps arrested speciation event.

ACKNOWLEDGMENTS

We are grateful to Yuri M. Mizusawa for his valuable help in the laboratory and in sampling the bees. Paulo Henrique P. Gonçalves also helped sampling the bees. We thank Susy Coelho and Julie Lim for technical assistance. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (04/15801-0; 08/08546-4; 10/50597-5) and Australian Research Council. This work was developed in the Research Center on Biodiversity and Computing (BioComp) of the Universidade de São Paulo (USP), supported by the USP Provost's Office for Research.

Hybridation et introgression asymétrique entre *Tetragonisca angustula* et *Tetragonisca fiebrigi*

Espèce / sous-espèce / ADN mitochondrial / microsatellite/ Meliponini

Hybridisierung und asymmetrische Introgression zwischen *Tetragonisca angustula* und *Tetragonisca fiebrigi*

Art / Unterart / mitochondriale DNA / Mikrosatelliten / Meliponini

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