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Research Article

Genetic divergence in *Tetragonisca angustula* Latreille, 1811 (Hymenoptera, Meliponinae, Trigonini) based on rapd markers

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Abstract

One of the commonest neotropical stingless bees is *Tetragonisca angustula* (Latreille, 1811), popularly known in Portuguese as *jataí*, which occurs in variable nesting sites from Mexico to Argentina. We used 18 primers to generate 218 RAPD markers which we used to determined the genetic distance between *T. angustula* populations from 25 localities in three different Latin America countries, using *Tetragonisca weyrauchi* from the Brazilian state of Acre and the common honey bee (*Apis mellifera*) as outgroups. Genetic distance, calculated as the Percentage of Dissimilarity (14%), based on all markers divided the *T. angustula* population into eastern (group 1) and western (group 2) groups. However, we were able to separate the two groups by using only two primers that have generated five specific molecular markers. The eastern group consists of *T. angustula angustula* which occurs from Panama to the Brazilian states of Maranhão and northern Minas Gerais and has spread through the Brazilian Atlantic Forest as far as the southern Brazilian state of Santa Catarina. Group 2 is made up of *T. angustula fiebrigi* which has a more southerly and western distribution, occurring only in the western Brazilian states of Mato Grosso and Mato Grosso do Sul as well as the west of some other Brazilian states (Goiás, Minas Gerais, São Paulo, Paraná and Santa Catarina) and northeastern Argentina.

Key words: Tetragonisca angustula, genetic divergence, molecular markers, UPGMA.

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Introduction

One of the most common neotropical stingless bees is *Tetragonisca angustula* (Latreille, 1811) (Hymenoptera, Meliponinae), popularly known in Portuguese as *jataí*, which are generalist foragers and more efficient native pollinators of the American flora. Although *T. angustula* is widely distributed in the Americas from the south of Mexico to Argentina, it has not been observed in Andean cordillera, the scrubland (caatinga in Portuguese) of northeastern Brazilian and some regions of the Amazon.

There are two subspecies of *T. angustula* which can be separated morphologically, *T. angustula angustula* (Latreille) with a black mesepisternun and *T. angustula fiebrigi* (Schwarz) with a yellow mesepisternun (Castanheira, 1995). According to Nogueira-Neto (1970), *T. a. angustula* is distributed as described above (i.e. from Mexico to Argentina with some exceptions) while *T. a. fiebrigi* is restricted to sites in southern Brazil (the Paraná river valley in São Paulo state, part of the state of Paraná and the state of Santa Catarina) and Argentina and Paraguay.

The biological, behavioral, morphological and biochemical aspects of *T. angustula* have been studied since the beginning of the twentieth century. Castanheira (1995) investigated 228 *T. angustula* colonies from five Brazilian states (Mato Grosso, Bahia, Minas Gerais, São Paulo and Paraná) for genetic differences in six enzyme systems and found variation at the hexokinase, glycerol-3-phosphate dehydrogenase and malate dehydrogenase *loci*, the correlation between the degree of subspecies hybridization in the São Paulo populations and the frequency of the hexokinase *Hk88* allele indicating that this allele is a marker for the *T. a. fiebrigi* subspecies. Diniz-Filho *et al.* (1998) studied geographical variation in morphology based on fifteen morphometric characters in eight local populations occurring in western-central and southeastern Brazil. These au-

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thors found significant differences in all the characters analyzed, with body-size showing a north-south clinal variation in which larger bees occurred mainly in the south, this being attributable to adaptation to a cooler climate (*e.g.* Bergmann's rule).

The study described in this paper used polymerase chain reaction - random amplified polymorphic DNA (PCR-RAPD) markers (Williams *et al.,* 1990; Welsh and McClelland, 1990) to investigate the relationship between different *T. angustula* populations, the results providing interesting insights into future research on this species.

Material and Methods

Bee samples and DNA extraction

We sampled worker bees from 26 *T. angustula* L. colonies from 25 sites (Table 1), the colony representing Pilar do Sul was in fact situated at Ribeirão Preto where it had been maintained for three years prior to sampling. For

Table 1 - Origin of the *Tetragonisca angustula* specimens studied and their identification based on the morphological characteristics given by Nogueira Neto (1970) as compared with RAPD markers.

Origin of the bees*			Subspecies	
City	State or province	Map Reference	Based on morphological data	Based on RAPD markers
Panama:				
Panama City	Curundu	08°57N, 79°32W	*T. a. angustula L.*	*T. a. angustula L.*
Brazil:				
Barra do Corda	Maranhão	05°30S, 45°15W	*T. a. angustula*	*T. a. angustula*
Mirador	Maranhão	06°21S, 44°22W	*T. a. angustula*	*T. a. angustula*
Porangatu	Goiás	13°26S,°49°10W	*T. a. angustula*	***T. a. fiebrigi S.***
Campinápolis	Mato Grosso	14°30S, 52°53W	***T. a. fiebrigi S.***	***T. a. fiebrigi***
São Francisco	Minas Gerais	15°57S, 44°51W	*T. a. angustula*	*T. a. angustula*
Bocaiúva	Minas Gerais	17°06S, 43°48W	*T. a. angustula*	*T. a. angustula*
Rio Verde	Goiás	17°49S, 50°55W	*T. a. angustula*	***T. a. fiebrigi***
Uberlândia[1]	Minas Gerais	18°54S, 48°16W	*T. a. angustula*	***T. a. fiebrigi***
Uberlândia[2]	Minas Gerais	18°54S, 48°16W	*T. a. angustula*	***T. a. fiebrigi***
Grupiara	Minas Gerais	18°30S, 47°44W	*T. a. angustula*	***T. a. fiebrigi***
Ladário	Mato Grosso do Sul	19°01S, 57°35W	***T. a. fiebrigi***	***T. a. fiebrigi***
Campina Verde	Minas Gerais	19°31S, 49°28W	*T. a. angustula*	***T. a. fiebrigi***
Araxá	Minas Gerais	19°35S, 46°56W	*T. a. angustula*	*T. a. angustula*
Domingos Martins	Espírito Santo	20°22S, 40°40W	*T. a. angustula*	*T. a. angustula*
Canaã	Minas Gerais	20°41S, 42°36W	*T. a. angustula*	*T. a. angustula*
Ribeirão Preto	São Paulo	21°11S, 47°48W	*T. a. angustula*	***T. a. fiebrigi***
Pedreira	São Paulo	22°43S, 46°55W	*T. a. angustula*	*T. a. angustula*
Maricá	Rio de Janeiro	22°55S, 42°49W	*T. a. angustula*	*T. a. angustula*
Pilar do Sul[3]	São Paulo	———[3]	*T. a. angustula*	***T. a. fiebrigi***
Curitiba	Paraná	25°25S, 49°17W	*T. a. angustula*	*T. a. angustula*
Prudentópolis	Paraná	25°12S, 50°58W	***T. a. fiebrigi***	***T. a. fiebrigi***
Blumenau	Santa Catarina	26°55S, 49°05W	*T. a. angustula*	*T. a. angustula*
Argentina:				
Aristóbulo del Valle	Misiones	27°06S, 54°55W	***T. a. fiebrigi***	***T. a. fiebrigi***
Posadas Misiones	Misiones	27°23S, 55°54W	***T. a. fiebrigi***	***T. a. fiebrigi***
Cerro Azul	Misiones	27°37S, 55°29W	***T. a. fiebrigi***	***T. a. fiebrigi***

*For each country the sites are ordered in terms of increasing latitude.
Bold-faced type is used only to make it easier to read the table.
[1,2]Different samples of same colony.
[3]Sample collected from a three year colony which was then kept at Ribeirão Preto.

outgroups we used two *Tetragonisca weyrauchi* (Schwarz) colonies from the Brazilian state of Acre (09°58'S, 67°48'W) and an *Apis mellifera* colony from Uberlândia (18°54'S 48°16'W). In all cases, the samples consisted of a worker bee collected at the entrance of each colony and kept in absolute alcohol at -80 °C until DNA extraction (Post *et al.*, 1993).

Each bee was washed three times in buffer (TrisCl 0.01 M, pH 8.0; NaCl 0.1 M and $MgCl_2$ 0.001 M) for 15 min (Dowton and Austin, 1994) and the DNA extracted as using a modified Shuster *et al.* (1992) protocol, *i.e.* incubation with proteinase K and Rnase, phenol-chloroform extraction and DNA precipitation using 70% ethanol.

RAPD-PCR amplification and separation and visualization of products

We used eleven decamer short primers (OPA 03, 09, 10, 13; OPF 13; OPL 04, 11, 13; OPO 06; OPV 07 and 10) from Operon Technologies, USA and seven 20 to 24 mer long primers (GOU 07, 10; MAU 101, B1, B2, 402 and 801) which we synthesized in our laboratory. Each amplification was performed in a total of 25 µL of reaction mixture consisting of 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 100 mM of each dNTP (dCTP, dATP, dGTP, dTTP), 10 pmoles of primer, 1U of *Taq* DNA polymerase and 10 ng of bee genomic DNA. Reactions were performed in a PTC-100 thermal cycler (MJ Research) for three cycles of 94 °C for 1 min, 35 °C for 1 min and 72 °C for 2 min, followed by 34 cycles of 94 °C for 10 s, 40 °C for 20 s and 72 °C for 2 min with a final cycle of 72 °C for 5 min. A negative control reaction was prepared for each primer used in all PCR amplifications to identify contamination of reactions with non-target template DNA. All amplification reactions were repeated twice in order to test reproducibility.

Amplified products were separated by electrophoresis in 1% (w/v) agarose gel for 1.5 h at 0.5 V cm^{-2} using 0.5X TBE (tris- EDTA borate) buffer, stained with ethidium bromide and photographed using a VDS Image Master (Pharmacia Biosciences). A DNA molecular weight marker (Gibco-BR) was also included.

Amplicon analyses

Only those fragments that were consistently reproduced were used. A binary matrix was produced in accordance with the presence (1) or absence (0) of bands, the fraction of bands matching in two bees (M) being calculated using the formula $M = N_{AB}/N_T$ where N_{AB} equals the total number of matches (*i.e.* both bands absent or both bands present) in individuals A and B and N_T is the total number of fragments scored. Genetic distance (GD) was calculated as GD = 1 - M and cluster analyses performed on the GD values using an unweighted pair-group method with averages (UPGMA) algorithm, the resultant dendrograms being generated using the version 4.5 of the Statistica program (Puterka *et al.,* 1993).

Results and Discussion

Cluster analysis of *T. angustula* genetic distance values

The RAPD method has been reported to be an efficient tool to differentiate geographically and genetically isolated populations, and has been used to verify the existence of populations of species that have arisen either through genetic selection under different environmental conditions or as a result of genetic drift (Fuchs *et al.*, 1998).

Samples of *T. angustula* analyzed using RAPD profiles generated by 18 primers resulted in 218 intense and reproducible polymorphic fragments. The cluster analysis dendrogram (Figure 1) divided the *T. angustula* population into two groups with 14% of genetic dissimilarity. One group clustered 12 populations (Panama, Mirador, Barra do Corda, Domingos Martins, Araxá, Bocaiúva, Canaã, São Francisco, Curitiba, Blumenau, Maricá and Pedreira), while the other group (Porangatu, Rio Verde, Grupiara, Campina Verde, Uberlândia 1 and 2, Campinápolis, Ladário, Ribeirão Preto, Pillar do Sul, Prudentópolis, Posadas, Aristóbulo del Valle and Cerro Azul) consists of 13 populations from the western side of South America. The genetic distance between groups 1 and 2 suggests that this species is undergoing genetic differentiation, a process which can be driven by ecological, evolutionary or historical factors. Genetic differentiation of *T. angustula* between the different groups of sampling sites is to be expected because the sites extend over a wide geographical area and are subject to different environmental factors, such factors including the temperature gradient from the equator to Argentina, vegetation patterns which vary from rain forest to cerrado (savanna-like vegetation) and the pampas of southern Brazil and Argentina, differences in rainfall and other, unknown, environmental factors and processes.

Group 1 (*T. a. angustula*) occurs in the east of the collection area, from Panama to southern Brazil, while Group 2 (*T. a. fiebrigi*) was distributed in the Brazilian states of Mato Grosso and Mato Grosso do Sul, the western parts of the Brazilian states of Goiás, Minas Gerais, São Paulo and Paraná, and northeastern Argentina (Figure 2). Our RAPD marker study shows that 56% of the 25 geographical sites were colonized by *T. a. fiebrigi*, showing that this subspecies is more widely distributed than is apparent when this subspecies is identified by mesepisternun color which identified *T. a. fiebrigi* in only 24% of the sites (Table 1). Previously (Nogueira-Neto, 1970), morphological data suggested that *T. a. fiebrigi* occurred only in southern Brazil in parts of São Paulo, Paraná and the River Paraná valley and the state of Santa Catarina, and in Paraguay and Argentina.

The wide geographic range of *T. a. fiebrigi* as suggested by RAPD markers may be the result of intraspecific hybridization and/or *T. a. fiebrigi* having no established allopatric limits.

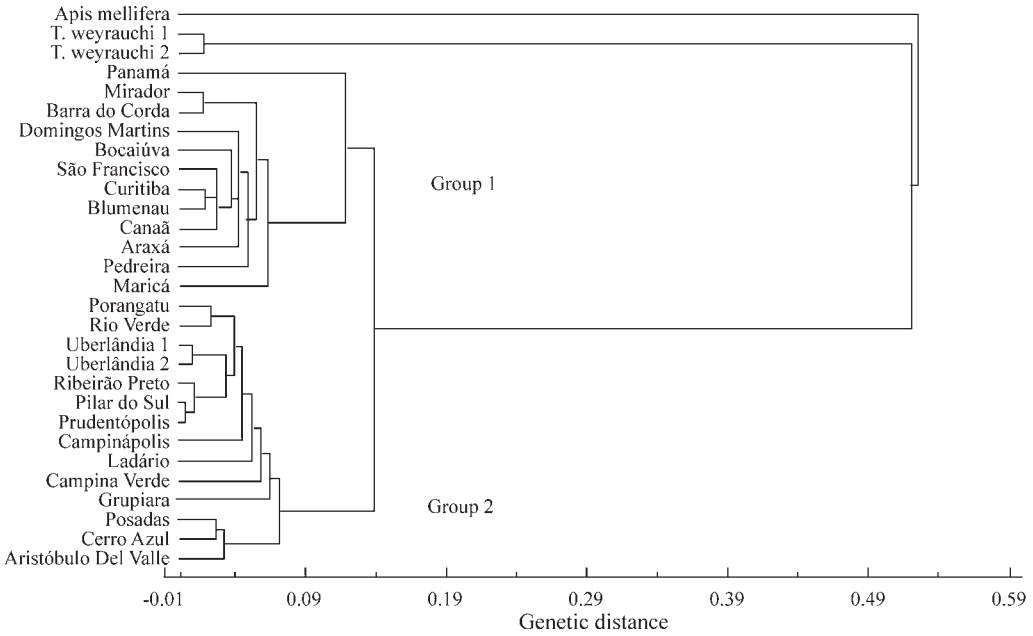

Figure 1 - Genetic distance based on percentage disagreement of 218 RAPD markers obtained from 18 random primers grouped by the unweighted pair group method with averages (UPGMA) for 29 genotypes (26 *T. angustula,* two *T. weyrauchi* and one *A. mellifera)*.

Figure 2 - Geographic distribution of the two *T. angustula* clusters: Group 1 (red) includes: 1- Panama, 2- Mirador, 3- Barra do Corda, 8- Domingos Martins, 9- Maricá, 10- São Francisco, 11- Bocaiúva, 15- Araxá, 17- Canaã, 19- Pedreira, 21- Curitiba and 23- Blumenau. Group 2 (black): 4- Porangatu, 5- Rio Verde, 6- Campinápolis, 7- Ladário, 12 and 13- Uberlândia, 14- Grupiara, 16- Campina Verde, 18- Ribeirão Preto, 20- Pilar do Sul, 22- Prudentópolis, 24- Posadas, 25- Aristóbulo del Valle, and 26- Cerro Azul. The asterisk indicates the sample collected from a three-year-old colony from Pilar do Sul which had been maintained for three years prior to sampling in Ribeirão Preto.

The occurrence of *T. a. fiebrigi* could be the result of intraspecific hybridization. In our RAPD maker study there was only one possible intraspecific hybridization event, which can be seen in Figure 2 where the Pilar do Sul genotype (allocated to group 2 (*T. a. fiebrigi*) by our RAPD analysis) appears to be introgressing into group 1 (*T. a. angustula*). As explained in the materials and methods, the Pilar do Sul colony (morphologically *T. a. angustula*) was actually in Ribeirão Preto where it had been maintained for three years prior to sampling while the other colony sampled (also morphologically *T. a. angustula*) had always been in Ribeirão Preto, and it may have been that intraspecific hybridization occurred between these two colonies to produce *T. a. fiebrigi*. Intraspecific hybridization would be expected to result in co-dominant bands but this did not occur in this case, probably because RAPD markers usually yield dominant bands. Another way of demonstrating intraspecific hybridization would be by monitoring the introgression of RAPD markers in haplodiploid crossings, which also was not the case. However, Castanheira (1995) has demonstrated that rare alleles have been constantly introduced into the Ribeirão Preto *T. a. angustula* population due to importation of colonies of bees from other sites by beekeepers and researchers, isozyme markers of *T. a. fiebrigi* having been found in natural populations of Ribeirão Preto bees.

The zone of allopatry between these subspecies in the interior of South America has never been clearly established. The work related to the presence of subspecies of *T. angustula* dates from the beginning of the 20th century and

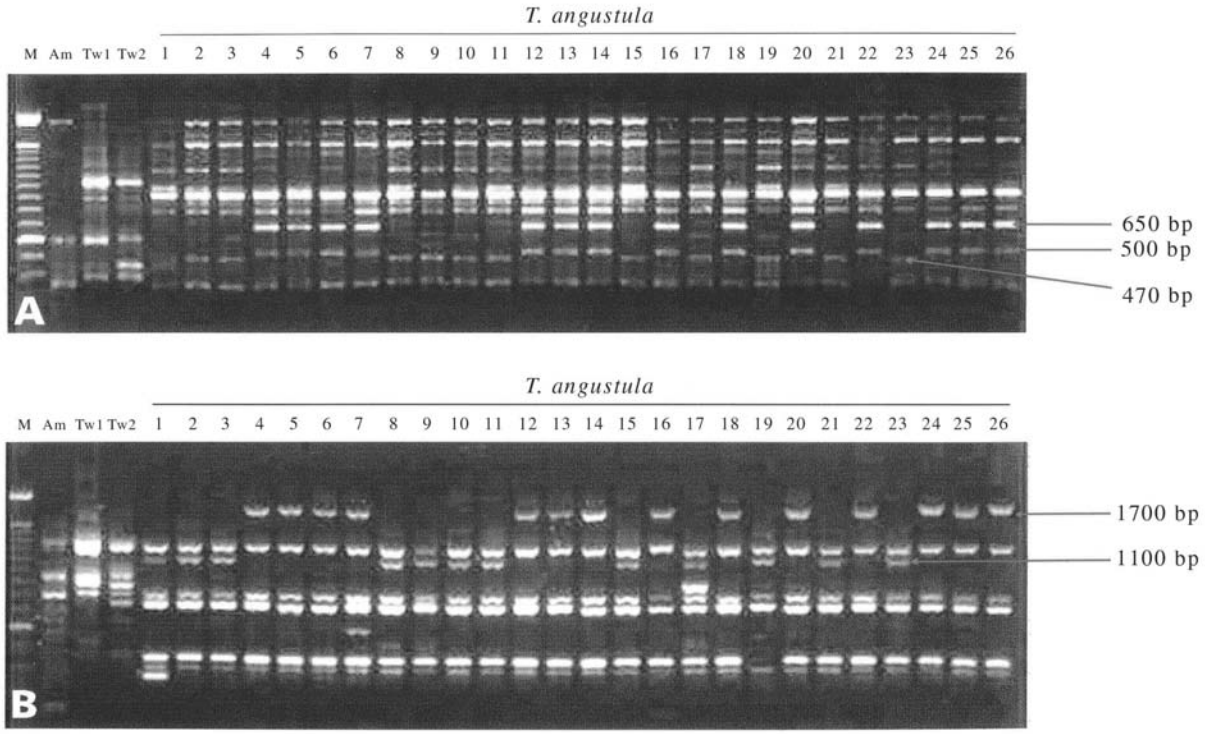

Figure 3 - Agarose gel (1%) electrophoresis of amplicons obtained with the MAU-B2 primer **(A)** and the OPL11 primer **(B)**. M = DNA Molecular weight Marker (Gibco-BR), Am- *Apis mellifera,* Tw1 and 2- *T. weyrauchi*. Lanes 1 to 26 are *T. angustula* samples: 1- Panama, 2- Mirador, 3- Barra do Corda, 4- Porangatu, 5- Rio Verde, 6- Campinápolis, 7- Ladário, 8- Domingos Martins, 9- Maricá, 10- São Francisco, 11- Bocaiúva, 12 and 13- Uberlândia, 14- Grupiara, 15- Araxá, 16- Campina Verde, 17- Canaã, 18- Ribeirão Preto, 19- Pedreira, 20- Pilar do Sul, 21- Curitiba, 22- Prudentópolis, 23- Blumenau, 24- Posadas, 25- Aristóbulo del Valle, 26 - Cerro Azul. The arrows indicate the markers of groups 1 and 2.

has incorporated only one morphologic character for discrimination, the coloration of the mesepisternun. This seems unreliable because morphologic characters can be under quantitative, epistatic or pleiotropic genetic control, which can mask the geographic distribution of populations. The use of more discriminatory and complementary tools such as RAPD analysis and methods for the detection of enzymatic systems that reveal heterozygotes could be used to detect population polymorphisms and a systematic revision of the genus could clarify the results of more preliminary work. Our sampling procedure was too limited to establish range limits.

By removing specific markers belonging to the outgroups we produced two other dendrograms (not shown), one with all *A. mellifera* markers removed and the other with all *A. mellifera* and *T. weyrauchi* markers removed. However, neither of the dendrograms showed any alterations in the *T. angustula* genotype clusters for either of the groups, although the genetic distance between groups 1 and 2 slightly increased to 16.7% when the *A. mellifera* markers were removed and 24.5% when both the *A. mellifera* and *T. weyrauchi* markers were removed. It may be that the 24.5% genetic distance between *T. angustula* group 1 (*T. a. angustula*) and group 2 (*T. a. fiebrigi*) may be a more reliable distance estimate because the presence of data for other species (the outgroups) may influence the divergence analysis by minimizing distance within the same species.

Population markers

In our search for genetic markers we found two primers, MAU-B2 (GCCAGGCAGCA AGTTCTCAGTAAT) and OPL11 (ACGATGAGCC), each of which could differentiate between the two geographically well-characterized *T. angustula* clusters. The MAU-B2 primer (Figure 3-A) presents three informative polymorphic bands of approximately 470, 500 and 650 bp, with the 470 bp band identifying group 1 (*T. a. angustula*) and the 500 and 650 bp bands segregating together in group 2 (*T. a. fiebrigi*, found only in the southwest of the area studied). The OPL11 primer (Figure 3-B) was also able to distinguish the two *T. angustula* clusters based on two bands, a 1100 bp band occurring only in group 1 genotypes and a band of about 1700 bp present only in group 2 genotypes.

It was thus possible to distinguish between the two *T. angustula* groups using only two primers, a significant fact because RAPD markers are easy to obtain and make possible genetic evolutionary studies simpler to conduct. Suazo

et al. (1998) have also detected specific RAPD markers which are able to distinguish between *A. mellifera* races from Africa and Europe, while Waldschmidt *et al.* (2000) used the same technique to identify a DNA marker present in *Melipona quadrifasciata quadrifasciata* but absent in *M. q. anthidioides*.

The findings reported in this paper have important implications for future research on *T. angustula.* The characterization and monitoring of natural species, using other molecular markers such as microsatellites, single-strand conformation polymorphism (SSCP-PCR) and sequencing of conserved genes will be of great value in establishing a clear picture of the distribution and dynamics of *T. angustula* in neotropical regions.

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