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Mitochondrial polymorphism in *Tetragonisca angustula* and *Tetragonisca weyrauchi* (Apidae) in northern Brazil

L. Ronqui[1], S.A. Santos[2], K.F. Araujo[2], C.A. Mangolin[2], V.A.A. Toledo[3], and M.C.C. Ruvolo-Takasusuki[2]

[1] Departamento Interdisciplinar de Tecnologia e Ciências, Universidade Federal de Rondônia- Ariquemes, RO, Brasil
[2] Departamento Biotecnologia, Genética e Biologia Celular, Universidade Estadual de Maringá (UEM), PR, Brasil
[3] Departamento de Zootecnia, Universidade Estadual de Maringá (UEM), PR, Brasil

Corresponding author: M.C.C. Ruvolo-Takasusuki
E-mail: mccrtakasusuki@uem.br

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ABSTRACT. Bees are important pollinating agents and are an integral part of food production and plant maintenance. It is useful to investigate mitochondrial polymorphisms in bees to obtain information that may be relevant to conservation strategies. We examined mitochondrial haplotypes and evaluated genetic diversity and differentiation in the native stingless bees *Tetragonisca angustula* and *Tetragonisca weyrauchi* using PCR-restriction fragment length polymorphism. Worker bees were collected from nests in Rondônia state. After isolating DNA, analyses were performed using 10 pairs of heterologous primers for *T. weyrauchi*, amplifying various regions of mitochondrial DNA (mtDNA). Primers and restriction enzymes were used for the first time for *T. weyrauchi* but had already been used in other studies with *T. angustula*. Only four of the tested primers (primer 1 - ND2 and COI; primer 2 - COI; primer 8 - 16S and 12S, and primer 9 - COII) were used for further analyses. For restriction analysis of the amplified regions, 13 enzymes were tested. Primer 1 - (ND2, COI) allowed the

identification of a fragment approximately 2,400 bp in size in *T. angustula* and *T. weyrauchi*. Fragment cleavage was accomplished using EcoRI and EcoRV enzymes. An approximately 1,850 bp fragment was amplified in the two species using region (COI) primer 2. Using the EcoRV enzyme, cleavage was confirmed only in *T. angustula* individuals; however, it was observed in both species using the HinfI enzyme. Amplification of the region, using 16S and 12S - primer 8, generated two fragments (1,850 and 350 bp in size), and cleavage was observed in both species using EcoRV, RsaI, and PstI enzymes; however, the XbaI enzyme cleaved in *T. weyrauchi* alone. Amplification of DNA, using (COII) primer 9, generated a 1,000 bp fragment. The cleavage was performed using ClaI and HinfI enzymes in *T. angustula*. Bayesian inference analysis showed that mtDNA of *T. angustula* has a greater genetic variability than that of *T. weyrauchi*. The variations observed by the analysis of mtDNA using PCR-RFLP showed differences in mtDNA in the two species.

Key words: Stingless bees; Amazonia; PCR-RFLP; Bayesian inference

INTRODUCTION

Bees are insects belonging to the order Hymenoptera, from the superfamily Apoidea (Michener, 2000; Camargo and Pedro, 2013). They are known worldwide for the various benefits they provide, such as the ability of some species to produce honey and their natural role in pollination.

Stingless bees are among the most common pollinators in tropical environments and, in certain regions, are the dominant bees, visiting various cultures (Macías-Macías et al., 2009). They comprise a diverse group of insects, which includes over 400 species that show high variability in physiology, morphology, and size (Michener, 2000; Moure et al., 2007).

Four species make up the genus *Tetragonisca*: *Tetragonisca angustula*, *Tetragonisca fiebrigi*, *Tetragonisca weyrauchi*, and *Tetragonisca buchwaldi*. Three species (*T. angustula*, *T. fiebrigi*, and *T. weyrauchi*) are found in Brazil. The species *T. weyrauchi*, which has the most restricted distribution is found in the states of Mato Grosso, Rondônia, and Acre, while *T. angustula* has a distribution throughout the country (Camargo and Pedro, 2013).

However, the constant deforestation and extractivism by the honey gatherers are increasing the pressure on this important natural resource. Thus, it is necessary to use advanced strategies in order to help its conservation. Bees of the genus *Melipona* are especially sensitive to environmental disturbances, as they depend on trees for dwelling and food (Bruening, 2006 and Vossler, 2012).

Molecular markers have been used as an important tool in the conservation of wildlife species and/or those of economic interest, because they provide important data for population studies, such as estimate of genetic variability rate, endogeneity rate, difference between populations, gene flow among populations, and determination of effective population size (Müller et al., 2010 and Tavares et al., 2013).

Mitochondrial DNA (mtDNA) polymorphism analysis is a useful research approach to investigate genetic variability and has been successfully applied to study insect populations, including meliponini species (Francisco and Arias, 2010). Studies indicate a low genetic diversity in wild populations of Brazilian stingless bees (Costa et al., 2005; Arias et al., 2006; Tavares et al., 2007; Borges et al., 2010; Brito and Arias, 2010; Francisco and Arias, 2010). This low genetic diversity can have negative consequences for the long-term survival rate of the population and raises important questions for conservation programs. We examined the genetic diversity in *T. angustula* and *T. weyrauchi* from Rondônia State using PCR-RFLP.

MATERIAL AND METHODS

Bee Collection

To characterize the adult worker bees of *T. angustula* and *T. weyrauchi*), 20 specimens were collected at the entrance of 32 wild colonies. Twenty-two colonies belonged to *T. angustula* species collected in the following locations in Rondônia State - Brazil: Porto Velho (two colonies), Cacoal (two colonies), Ji-Paraná (two colonies), Jaru (two colonies), Cacaulândia (two colonies), Alto Paraíso (three colonies), Ariquemes (six colonies), and Monte Negro (three colonies) (Figure 1).

Tetragonisca *weyrauchi* workers (20 specimens per colony) were collected from 10 wild colonies in the municipality of Alto Paraíso (Rondônia State - Brazil) (Figure 1). The smaller number of sampled *T. weyrauchi* nests was a consequence of the smaller number found and their location (on top of trees approximately 30 m high).

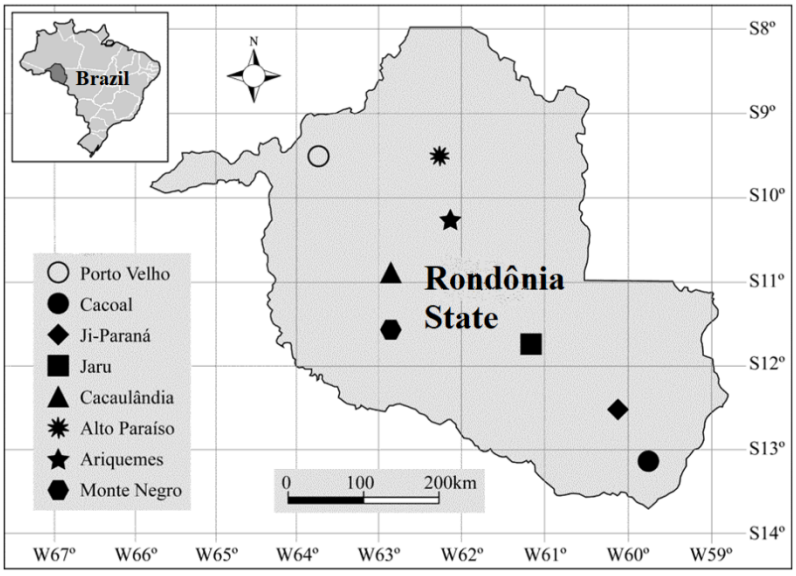

Figure 1. Map indicating the collection sites of *Tetragonisca angustula* and *Tetragonisca weyrauchi* samples in Rondônia state, Brazil.

The collections were carried out in accordance with the authorization number 29423-2 granted by the Chico Mendes Institute for Biodiversity Conservation - ICMBio through the System of Authorization and Information on Biodiversity - SISBIO.

Genomic DNA Extraction

The method employed to extract the total DNA was based on the modified method described by Yu et al. (1993). The thorax was individually homogenized in 1.5-mL tubes containing 300 mL of extraction buffer (200 mM Tris-HCl (pH 8.0), 0.5% SDS, 250 mM NaCl, 50 mM EDTA, and 100 mg/mL proteinase K (Invitrogen). After 1 h of incubation at 65°C, the material was centrifuged for 10 min at 10,000 × g, and the supernatant was transferred to a new tube, adding an equal volume of chloroform:isoamyl alcohol (24:1), followed by centrifugation at 10,000 × g for 10 min and transfer of the supernatant to a new tube. This procedure was performed twice. After homogenization and centrifugation for phase separation, the upper phase was transferred to a new tube. The DNA was precipitated by the addition of 250 µL of cold isopropanol and incubated at −20°C overnight. The precipitated DNA was separated by centrifugation at 10,000 × g for 10 min. After discarding the supernatant, the precipitate was washed with 1 mL of 70% cold ethanol and allowed to dry at room temperature. After drying, the DNA was resuspended for 2 h in 30 µL of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), treated with RNAse (10 µg/mL), kept for 2 h at room temperature, and stored at −20°C. The DNA quantification was evaluated using a Picodrop Microliter UV/Vis Spectrophotometer - Zenon Bio with a wavelength range of 230–850 nm.

PCR-RFLP

Initially, tests were carried out with 10 heterologous primers originally described by Hall (1994) and Smith and Simon et al. (1994) (Table 1), according to the methodology used by Francisco et al. (2001). PCR-RFLP was performed on five specimens from each nest.

The amplification conditions were based on the methodology described by Barni et al., (2007) with modifications in the annealing temperatures of the primers. For a reaction volume of 20 µL, 1× Tris-HCl buffer (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 2.5 mM of $MgCl_2$, 0.25 µM of each primer, 2.5 mM of each dNTP (dATP, dTTP, dCTP, and dGTP), two units of Taq Platinum DNA polymerase (Invitrogen), and 10 ng of template DNA was used. Biosystems, Veriti 384 thermal cycler was used and programmed with initial denaturation at 94°C for 5 min and final extension at 64°C for 10 min. Thirty-five cycles were performed, with each cycle at 94°C for 1 min, at specific annealing temperature for each primer for 1 min, and 20 s, and at 64°C for 2 min.

The amplification products were separated on 1% agarose gel at 60 V using 0.5× TBE buffer (Tris-borate, EDTA pH 8.0) and stained with ethidium bromide (0.5 µg/mL). Fragments were visualized under ultraviolet light, and the image was captured using the L-Pix HE photo-documentation system. A DNA molecular weight marker (100 bp, DNA ladder - Invitrogen) was used to determine the size of the generated fragments.

For the restriction analysis, the amplified mtDNA fragments were digested for 12 h with the following enzymes: EcoRI, EcoRV, HindIII, HinfI, RsaI, PstI, XbaI, HaeIII, ClaI,

XhoI, BglII, PvuII, and ScaI (Invitrogen). For each cleaving reaction, 3 µL of the PCR product, 3 U of restriction enzyme, 1× enzyme buffer, and 14.7 µL Milli-Q water was used. The results of the digestion were analyzed on 1% agarose gel at 60 V using 0.5× TBE buffer and stained in an ethidium bromide bath (0.5 µg/mL). The fragments of the cleavage were visualized under ultraviolet light, and the image was captured using the L-Pix HE photo documentation system. A DNA molecular weight marker (100 pb, DNA ladder) was used to determine the size of the generated fragments.

Table 1. Primer pairs used to amplify mitochondrial DNA (mtDNA) of *Tetragonisca angustula* and *Tetragonisca weyrauchi* and annealing temperature (N- number of *primers*, R - References, T - Temperature °C).

N	Name	Sequence (5`→3`)	R	Gene	T
1	mtD2 mtD9	GCTAAATAAGCTAACAGGTTCAT CCCGGTAAAATTAAAATATAAACTTC	1	ND2, COI	45
2	mtD7 COI-IIR	GGATCACCTGATATAGCATTCCC GATCAATATCATTGATGACC	1,2	COI	46
3	mtD19 mtD22	GAAATTTGTGGAGCAAATCATAG TCAACAAAGTGTCAGTATCA	1	ATPases (8,6), COIII	47
4	5612R tPheF	GAAATTAATATAACATGACCACC GCGTAATATTGAAAATATTAATGA	3	COIII, ND3	47
5	mtD24 mtD28	GGAGCTTCAACATGAGCTTT ATT.ACACCTCCTAATTTATTAGGAAT	1	ND4, ND6, CytB	44
6	mtD26 mtD30	TATGTACTACCATGAGGACAAATATC GTAGCATTTTTAACTTTATTAGAACG	1	CytB, ND1	45
7	Mel 3 16SF	TAAAGTTAAAAAAGCAACTC CACCTGTTTATCAAAAACATGTCC	3,2	16S	44
8	16SR mtD36	CGTCGATTTGAACTCAAATCATG AAACTAGGATTAGATACCCTATTAT	2,1	16S, 12S	45
9	MtD18 COI-IIF	CCACAAATTTCTGAACATTGACCA TCTATACCACGACGTTATTC	1,2	COII	47
10	Seq 18 8467F	GAACTATCAATTTGATATTG GGAATTTTTTTTTGAATGAAA	3	ND4, ND5	46

[1](Simon et al., 1994); [2](Hall and Smith, 1991); [3](Francisco et al., 2001).

Data Analysis

The analyses of the molecular data involved the interpretation of mtDNA fragments. The identity and genetic distance of Nei (1978) was calculated using the Popgene 1.32 program (Yeh et al., 1999). The GeneAIEx 6.5 program (Peakall and Smouse, 2006) was used for Molecular Variance Analysis (AMOVA) using phiPT statistics (ΦPT). In order to detect the variation patterns within and between the two species of *Tetragonisca* analyzed, Principal Coordinate Analysis (PCoA) (GeneAIEx 6.5 program, Peakall and Smouse, 2006) was used to find and plot the variations in a multivariate data set.

The estimate of the actual number of populations was made according the method described by Evanno et al. (2005) using the delta statistic K = Ln (P (X / K)) between

successive K values (number of populations) and X = probability of observing the data. The estimation was made with K values varying from 2 to 8, and the K value used was the one with the highest delta K value. This estimation was performed using the structure 2.3.4 software (Pritchard et al., 2000) and Harvester structure (http://taylor0.biology.ucla.edu/struct_harvest/). The burn-in was fixed at 3,000 repetitions and the Monte Carlo Method via Markov chains (MCMC) at 30,000. The number of interactions established was 10.

RESULTS

Analysis of mtDNA regions with PCR-RFLP technique

This is the first study carried out using PCR-RFLP in *T. weyrauchi* and the first description of heterologous primers for this species of stingless bee.

Only four of the primers tested for amplification presented reproducible fragment patterns (primers 1, 2, 8 and 9), and were used in the analyses. The other pairs of primers amplified but did not present reproducible fragment patterns.

Primer 1 (ND2, COI)

Amplification of mtDNAs using primer 1 enabled the identification of a fragment of approximately 2,400 bp in size in *T. angustula* and *T. weyrauchi*. The 2,400bp fragment digested with the EcoRI enzyme was only cleaved in *T. weyrauchi*, allowing to identify a pattern with two bands, one with approximately 1,800 bp and the other with 400 bp.

The approximately 2,400-bp fragment was also cleaved by EcoRV in *T. weyrauchi*, generating two fragments of 1,700 bp and 700 bp in size. The cleaving using Rsal enzyme in the same region resulted in two fragments 2,000 and 400 bp in size in *T. weyrauchi*.

Primer 2 (COI)

Primer 2 amplified in both species (*T. angustula* and *T. weyrauchi*). The fragment was approximately 1,850 bp. Cleaving using the EcoRV enzyme occurred only in individuals of *T. angustula* and generated two fragments 1,000 and 850 bp in size. No cleaving occurred in individuals of *T. weyrauchi*. However, cleaving using the HinfI enzyme in both species generated three fragments approximately 900, 400, and 300 bp in size.

Primer 8 (16S, 12S)

The amplified mtDNA segment for primer 8 generated two fragments, one of approximately 1,850 bp and the other of 350 bp in size, which were observed in all the individuals of both species. Cleaving was observed in both species after digestion with the EcoRV, RsaI, and PstI enzymes. The cleaving using EcoRV enzyme resulted in two

fragments 950 and 900 bp in size. Cleaving occurred using the Rsa1 enzyme for both *T. angustula* and *T. weyrauchi* and generated three fragments of approximately 700, 600, and 500 bp in size. Cleaving using the PstI enzyme for both species generated fragments of approximately 1,300 and 550 bp in size. Cleaving using the XbaI enzyme was only observed in *T. weyrauchi* and generated two fragments of 1,400 and 450 bp in size.

Primer 9 (COII)

The amplification of the DNAs using primer 9 in *Tetragonisca* species resulted in a 1,000-bp fragment. Cleaving using the ClaI enzyme resulted in two fragments, one of approximately 800 bp and one 200 bp in size. Digestion with the HinfI enzyme also generated two fragments, one of approximately 600 bp and one of 400 bp in size. No cleaving occurred in *T. weyrauchi* samples.

Population Genetics

For the four primers used for amplification, cleaving was successful using seven restriction enzymes, which allowed the detection of 11 PCR-RFLP loci. AMOVA was run using the phiPT value, which was 1.00. This value indicated that all variations occurred within the two species.

The PCoA showed that a single coordinate (axis one) was responsible for 76.01% of the variance among the formed groups. Axis 2 was represented by 16.76% and axis 3 by 3.64%.

The genetic distance value (Nei, 1978) observed between *T. weyrauchi* and *T. angustula* was 1.0116. The Bayesian inference analysis using the MCMC chain showed a similar result to that obtained with the analysis using the genetic distance of Nei (1978). As shown in Figure 2, numbers 1 and 2, corresponding to *T. angustula* and *T. weyrauchi*, were separated (estimated K = 5), indicating that these species had different patterns of mtDNA markers.

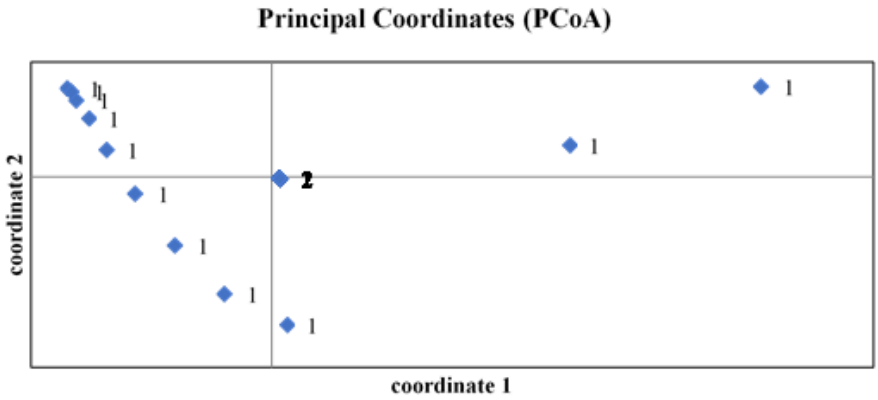

Figure 2. Principal Coordinates Analysis (PCoA). Numbers 1 and 2 represent Eigen Values in coordinates 1 and 2 (GeneAIEx 6.5 program, Peakall and Smouse, 2006).

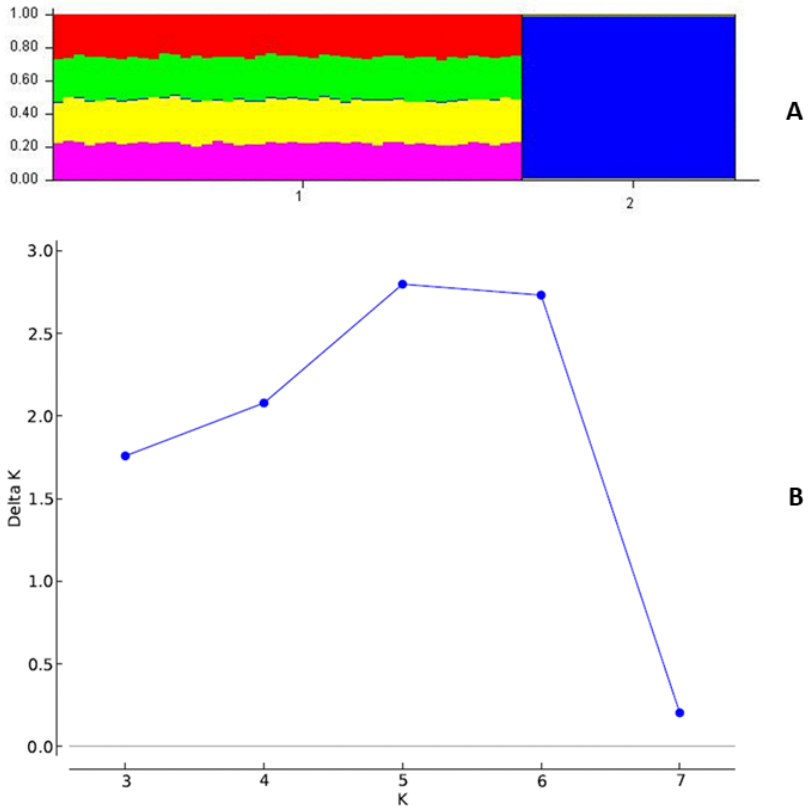

Figure 3. A: Bar graph obtained by Bayesian inference (Markov Chain Monte Carlo) with the estimated K = 5. B: Estimated number of populations based on the Evanno *et al*. (2005) method. Numbers 1 = *Tetragonisca angustula*, 2= *Tetragonisca weyrauchi*. The colored segments have lengths proportional to each of the K inferred groups that are represented in B.

DISCUSSION

Using ND2, COI (primer 1) in *T. angustula* and *T. fiebrigi* collected in three Brazilian states, Santos et al. (2015) observed polymorphism in samples of *T. angustula* from Porto Velho city (Rondônia State), where some individuals amplified a fragment 900 bp in size.

According to Santos et al. (2015), only one queen originates all the workers present in the *Tetragonisca* nest. Therefore, no variation in worker mtDNA was detected. The authors suggested that there was probably a mutation in this region of the queen's mtDNA, and the binding site of primer 1 amplifying the 900-bp fragment was altered, making amplification impossible. The *T. angustula* bees present in Rondônia are geographically isolated from those farther south (Paraná and São Paulo states), probably isolating such a mutation.

The results of our study were consistent with those reported by Santos et al. (2015), as the *Tetragonisca* samples amplified for the ND2, COI (primer 1) region, approximately 2,400 bp in size, and cleavage occurred using the same restriction enzymes (EcoRI and EcoRV) in *T. angustula* from the state of Rondônia, except for the cleavage using the RsaI enzyme, which occurred only in *T. weyrauchi*.

The ND2, COI (primer 1) region was also evaluated by Barni et al. (2007) in *Melipona rufiventris* and *Melipona mondury*. Amplification of a 2,200-bp fragment was observed, and digestion using EcoRI generated two fragments, 1,500 and 700 bp in size. Digestion with HinfI showed polymorphic fragments in different species, with a 1,600-bp fragment and a 600-bp fragment for *M. mondury* and a 1,850-bp fragment and 350-bp fragment for *M. rufiventris*. Thus, the same fragment amplified in different species may result in different fragments when cleaved.

The 1,850-bp fragment resulted from the amplification of (COI) primer 2 region observed in *T. angustula* and *T. weyrauchi* was also observed by Santos et al. (2015) in *T. angustula* and *T. fiebrigi*. However, these authors observed the cleaving of this region using two restriction enzymes (EcoRV and HinfI). Whereas, in *T. weyrauchi*, the cleaving occurred only using the HinfI enzyme. This same region in *M. mondury* and *M. rufiventris* amplified by Barni et al. (2007) was 1,900 bp in size in both species, and cleaving occurred only using the HinfI enzyme.

As described by Santos et al. (2015), the sequence (16S, 12S) primer 8 also generated two fragments, one approximately 1,850 bp and the other 350 bp in size; however, cleaving of the fragments yielded different results. Santos et al. (2015) observed the cleaving of the 1,850-bp fragments using the EcoRV, PstI, RsaI, and XbaI enzymes. On the other hand, in the samples of *T. angustula* and *T. weyrauchi*, cleaving using the XbaI enzyme was only observed in *T. weyrauchi*, generating fragment 1,400 and 450 bp. Barni et al. (2007) also detected a 1,850-bp fragment for the same amplified segment in *M. mondury* and *M. rufiventris*, but there was no cleaving using the EcoRI, EcoRV, HindIII and HinfI enzymes.

According to Santos et al. (2015), the (COI) primer 9 sequence presented two amplification patterns for *T. angustula*, depending on the location. The first pattern had a fragment with 1,000 bp and the had two fragments, 1,000 and1,100 bp. The 1,000 bp pattern cleaved with the ClaI enzyme, resulting in a haplotype with two fragments, one approximately 800 bp and the second other 200 bp. Restriction with HinfI also generated two fragments, approximately 600 and 400 bp. This was not consistent with that observed for *T. angustula* and *T. weyrauchi* from the State of Rondônia, which presented only the 1,000 bp pattern. In the *T. weyrauchi* samples, no restriction fragments were observed.

The amplified loci for *T. angustula* and *T. weyrauchi* were common to those amplified in the study of Santos et al. (2015), but the success in cleaving was lower (seven enzymes), while Santos et al. (2015) recorded eight enzymes. Their AMOVA analysis showed that 83% of the variation occurred between populations and 17% within populations, while in our study 100% of the variation occurred within the populations of *T. angustula* and *T. weyrauchi* from Rondônia.

In the state of Santa Catarina, Brazil, a study of mitochondrial genes was carried out on 138 colonies of *T. angustula* and 72 colonies of *T. fiebrigi*, and large genetic differences were observed, as four enzymes showed different restriction patterns that allowed the separation of subspecies (Koling and Moretto, 2010).

In another study conducted using microsatellite loci of *T. angustula*, 15 out of 21 loci proved to be polymorphic. Primers were successfully used to amplify microsatellite loci in *T. fiebrigi*, *T. weyrauchi*, *Lestrimelitta maracaia*, and *Schwarziana quadripunctata* (Brito et al. 2009).

Bayesian inference analysis using the MCMC chain in the study of Santos et al. (2015) estimated the number of populations at K = 6. However, they considered that it was not possible to differentiate populations of *T. angustula* and *T. fiebrigi*. Contrary to these results, we were able to separate *T. angustula* and *T. weyrauchi* using these markers. In addition, Bayesian inference mtDNA analysis of Rondônia bee samples showed that *T. angustula* had mtDNA with wider genetic variability than that of *T. weyrauchi*.

Lower genetic variability observed in *T. weyrauchi* may also be due to the smaller number of nests analyzed and the collection sites. However, characteristics such as the wide distribution of *T. angustula*, found from Mexico to the south of Brazil (Camargo and Pedro, 2013) and restricted distribution of *T. weirauchi* found in Bolivia, Peru (Junin, Madre de Dios) and Brazil (Acre, Mato Grosso and Rondônia) (Camargo and Pedro, 2013), are probably related to their genetic diversity. Meliponiculture beekeepers commonly exchange colonies of *T. angustula*, taking them to different locations, probably interfering with the original distribution of this species and with its genetic variability.

According to Lozier and Zayed (2017), genomic tools can revolutionize research for bee conservation, including the identification of loci and desirable adaptive and economic characteristics such as those involved in susceptibility to disease.

The results of the study of Francisco et al. (2017) showed that the populations of *T. angustula* evaluated using mtDNA were highly differentiated, the analysis of the genetic structure of 17 populations in southern Brazil detected 73 haplotypes, and the diversity of mtDNA was low in nine populations.

CONCLUSIONS

Polymorphism detected using mtDNA analyses using PCR-RFLP of the two species of *Tetragonisca* bees showed that there were differences in mtDNA. *T. angusutla* and *T. weyrauchi* presented distinct patterns of mtDNA markers.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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