Genetic structure of the stingless bee *Tetragonisca angustula*

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Abstract

The stingless bee *Tetragonisca angustula* Latreille 1811 is one of the most widespread bee species in the Neotropics, distributed from Mexico to Argentina. However, this wide distribution contrasts with the low distance that females travel to build new nests whereas nothing is known about male dispersion. Previous studies of *T. angustula* were ambiguous concerning its genetic structure and were based only on nuclear markers and on small and/or limited sample size. Here we evaluate the genetic structure of several populations of *T. angustula* by using mitochondrial DNA and microsatellites. These markers can help us to detect differences in the migratory behavior between males and females. Our results showed that the populations were highly differentiated suggesting that both females and males are low dispersers. Therefore, its continental distribution might consist of several different taxa.

Key words. islands, Meliponini, microsatellites, mtDNA, population genetics

Introduction

The stingless bee *Tetragonisca angustula* Latreille 1811 is one of the most widespread bee species in the Neotropics, distributed from Mexico to Argentina (Silveira *et al.* 2002; Camargo & Pedro 2013). It is a small (4-5 mm in length), generalist and highly eusocial bee (Michener 2007) and highly adaptable to different nest sites. Colonies comprise up to 5,000 individuals (Lindauer & Kerr 1960), and are usually built in tree trunks or in wall cavities. It presents high rates of swarming and it is extremely successful in surviving in urban environments (Batista *et al.* 2003; Slaa 2006; Velez-Ruiz *et al.* 2013). In addition, *T. angustula* is one of the most cultivated stingless bees in Latin America (Nogueira-Neto 1997; Cortopassi-Laurino *et al.* 2006) and nest transportation and trading is very common among beekeepers.

In general, colony reproduction in stingless bees begins by workers searching for a new nest site within their foraging range (van Veen & Sommeijer 2000a). The daughter nests are established at most a few hundred meters from the “mother” nest (Nogueira-Neto 1997). After selecting the site, several workers begin to transport cerumen, propolis and honey from the “mother" nest to the new one (Nogueira-Neto 1997). This transport can last from few days (van Veen & Sommeijer 2000a) to few months (Nogueira-Neto 1997). The virgin queen leaves the "mother" nest accompanied by hundreds of workers (van Veen & Sommeijer 2000b). The next day the virgin queen flies out, mates with presumably one male (Peters *et al.* 1999; Palmer *et al.* 2002), returns to the nest and about a week later begins oviposition (van Veen & Sommeijer 2000b).

In contrast, little is known about stingless bee males reproductive behavior. After birth, they remain in the nest for two to three weeks (Cortopassi-Laurino 2007). They then leave the nest and never return. There are no data about the behavior of males during their period outside the nest. In laboratory, males can live up to six weeks (Velthuis *et al.* 2005).

Therefore, they likely have two to four weeks for dispersal and reproduction. It has been shown that reproductive aggregations are composed of males from hundreds of different, and not necessarily from nearby colonies, suggesting high male dispersal (Paxton 2000; Cameron *et al.* 2004; Kraus *et al.* 2008; Mueller *et al.* 2012).

Studies on the genetic structure of populations can help us better understand dispersal behavior and evolutionary history. Few population genetic studies focusing *T. angustula* have been conducted using molecular markers. Nonetheless the conclusions were ambiguous concerning gene flow and populations differentiation (Oliveira *et al.* 2004; Baitala *et al.* 2006; Stuchi *et al.* 2008). In addition, these studies have analyzed only nuclear markers (RAPD and isozymes) on limited sample size or distribution. This makes difficult to detect differences in the migratory behavior between males and females and to make general inferences about their evolutionary history.

Here our aim is to evaluate the genetic structure of several populations of *T. angustula* by using mitochondrial DNA (mtDNA) and microsatellites. Considering the high distribution of *T. angustula* and the commonness of nest transportation and trading, we expect low genetic differentiation among populations despite the low female dispersal capability.

Materials and methods

Sampling

We collected 1,002 *T. angustula* from 457 sites distributed in mainland and on islands in south/south-eastern Brazil (Table S1). Eleven islands all with arboreal vegetation and of area greater than 1.0 km^2 were selected, 10 being land-bridge islands isolated about 12,000 years ago (Suguio *et al.* 2005) and one sedimentary island (Ilha Comprida) which arose about 5,000 years ago (Suguio *et al.* 2003). The islands range in size from 1.1 to 451 km^2 and are 0.1 to 38

km from the mainland (Table S2, Fig. 1). Bees were sampled from nests (n = 125, one per nest) and flowers (n = 877) (Table S1). At the end, samples were grouped into 17 populations, being 14 on the mainland and three on islands (Figure 1).

We preserved the specimens in 96% ethanol for transport to the laboratory. DNA extraction followed the protocol described in Francisco *et al.* (2014). We dried the specimens at room temperature for 20 min prior to DNA extraction.

Mitochondrial DNA sequencing

Two mitochondrial genes were partially sequenced: cytochrome c oxidase subunit 1 (*COI*) and cytochrome b (*Cytb*). Details about amplification and sequencing are given in Francisco *et al.* (2014).

Microsatellite genotyping

The samples were genotyped for eleven microsatellite loci: Tang03, Tang11, Tang12, Tang17, Tang29, Tang57, Tang60, Tang65, Tang68, Tang70, and Tang77 (Brito *et al.* 2009). PCR conditions for each locus are given in Francisco *et al.* (2014). Electrophoresis, visualization and genotyping were performed according to Francisco *et al.* (2011).

MICRO-CHECKER 2.2.3 (van Oosterhout *et al.* 2004) was used to identify null alleles and scoring errors. COLONY 2.0.1.7 (Jones & Wang 2010) was used to determine whether individuals collected in the same plant or places nearby were related. Samples were excluded from our data set if matched the following three criteria: collected at sites distant less than 2 km, indicated as related by COLONY, and sharing a mtDNA haplotype. Overall, 722 *T. angustula* bees from 17 populations were deemed suitable for further genetic analyses (Table 1).

GENEPOP 4.1.2 (Rousset 2008) was used to verify Hardy-Weinberg equilibrium (HWE) in populations and loci and to detect linkage disequilibrium (LD). Markov chain was set for 10000 dememorizations, 1000 batches and 10000 iterations per batch. In cases of multiple comparisons *P*-values were corrected by applying Sequential Goodness of Fit test by the program SGOF 7.2 (Carvajal-Rodríguez *et al.* 2009). This method is advantageous over other correction methods because it increases its statistical power with the increasing of the number of tests (Carvajal-Rodríguez *et al.* 2009).

Genetic diversity

ARLEQUIN 3.5.1.3 (Excoffier & Lischer 2010) was used to calculate mtDNA haplotype (h) and nucleotide (π) diversity. GENALEX 6.5 (Peakall & Smouse 2006, 2012) was used to calculate microsatellite allelic richness (A) and expected heterozygosity (H_E). Since sample sizes were different, allelic richness was standardized by rarefaction (Ar) using the program HP-RARE 1.0 (Kalinowski 2005). Differences in Ar among populations were estimated by Mann-Whitney two-tailed U Test (Mann & Whitney 1947). Inbreeding coefficients (F_{IS}) were calculated for each population with 10000 permutations using ARLEQUIN.

Population differentiation and gene flow

MEGA 5.2.1 (Tamura *et al.* 2011) was used to calculate mtDNA's number of base substitutions per site from averaging over all sequence pairs between populations using the Kimura 2-parameter (K2p) model (Kimura 1980). Population pairwise θ values (an F_{ST} analogue, Weir & Cockerham 1984) were calculated with 10000 permutations by ARLEQUIN using microsatellite alleles. When heterozygosity is high, F_{ST} and its analogues may not be appropriate measures of genetic differentiation (Hedrick 2005; Jost 2008; Heller & Siegismund 2009). For this reason, Jost's D_{est} (Jost 2008) was calculated. This statistc is not

influenced by heterozygosity (Jost 2008) and is more appropriate for microsatellite data (Heller & Siegismund 2009). Global D_{est} was calculated with 9999 permutations for mtDNA and microsatellite data using GENALEX. Pairwise D_{est} was calculated only for microsatellite data. Mantel tests between genetic and geographical distances among populations were performed with 9999 permutations by GENALEX to verify isolation by distance for both molecular markers.

BAPS 6 (Corander *et al.* 2008; Cheng *et al.* 2013) was used to infer population structure using microsatellites and the geographic coordinates of the sampled individuals to spatially cluster them. It is a Bayesian analysis of genetic population structure that creates *K* groups of individuals based on the similarity of their genotypes. The program was initially ran 5 times for each of $K = 1$ to 17 and then 10 times for each of $K = 5$ to 14. These results were used for admixture analysis with 200 iterations to estimate the admixture coefficients for the individuals, 200 simulated reference individuals per population and 20 iterations to estimate the admixture coefficients of the reference individuals.

Estimates of rates and direction of current and/or recent migration (*m*) between populations were determined by the program BAYESASS 3 (Wilson & Rannala 2003) using microsatellites multilocus genotypes through Markov chain Monte Carlo (MCMC) techniques. We performed five independent runs with 10^7 MCMC iterations, burn-in of 10^6 iterations and sampling frequency of 2000. The delta values used were 0.25 (migration), 0.40 (allele frequencies) and 0.55 (inbreeding).

Assessment of population demography

To detect the occurrence of a recent bottleneck event we used microsatellite data to verify the occurrence of excess of heterozygosity in the populations using the program BOTTLENECK 1.2.02 (Piry *et al.* 1999). We used the two-phased model (TPM) of mutation which is

155 suggested as the most appropriate for microsatellites (Di Rienzo *et al.* 1994). The variance
156 among multiple steps was 12 and the proportion of stepwise mutation model in the TPM was
157 95% as suggested by Piry *et al.* (1999). Altogether 10000 iterations were performed. The
158 significance of such deviation was determined with a Wilcoxon sign-rank test. In addition, our
159 second approach was to verify if allelic frequencies presented a mode shift away from an L-
160 shaped distribution (Luikart *et al.* 1998) also using BOTTLENECK.
161
162
163 **Results**
164 *Island occurrences*
165 *Tetragonisca angustula* was found and collected on five of the 11 islands visited (Table 1).
166 However, only the samples from Ilha Grande, Ilha de São Sebastião and Ilha Comprida were
167 included in the analyses. The other collections were not included due to small sample size
168 (Ilha do Cardoso, $n = 1$), and to individuals being highly related with anecdotal reports of
169 introduced nests (Ilha de Santa Catarina, see Francisco *et al.* (2014)).
170
171 *MtDNA diversity*
172 The *COI* gene sequences were 417 bp long (GenBank accession numbers KF222891-
173 KF223893) and 32 haplotypes were identified. The *Cytb* sequences were 391 bp long
174 (KF223894-KF224896) and generated 43 haplotypes. Most differences among haplotypes
175 were synonymous substitutions, since the number of unique amino acid sequences were four
176 for *COI* and 15 for *Cytb*. We concatenated the nucleotide sequences (808 bp) for population
177 analyses.
178 The 722 concatenated sequences defined 73 haplotypes. Since h and π were positively
179 correlated ($r = 0.510$, $P = 0.036$, $n = 17$) we hereafter use π as our measure of mtDNA

diversity. Nucleotide diversity ranged from 0.0006 ± 0.0019 (Passa Quatro) to 0.0407 ± 0.0251 (Ilha Comprida) (Table 1).

There was a non-significant positive correlation between the size of the sampled area and mtDNA diversity (r = 0.135, P = 0.606, n = 17). The correlation between median elevation and mtDNA diversity was negative but non-significant (r = -0.428, P = 0.087, n = 17).

MtDNA differentiation

Population structure was high. Sixty-seven haplotypes out of the 73 were population-specific. We built a haplotype network where the frequency and distribution of haplotypes are shown (Figure S1). It is worth noting the 'star-pattern' centered on four haplotypes, the high number of exclusive haplotypes and the great number of nucleotide substitutions separating Porto União/Foz do Iguaçu from the others. The populations Teresópolis, Resende, Prudentópolis, Angra dos Reis, and Ilha Grande all had unique haplotypes.

Global D_{est} was 0.772 ($P < 0.001$) indicating a highly significant population structure. The highest K2p values were found for Porto União/Foz do Iguaçu with respect to all other populations (2.809% to 3.306%) (Table 2).

Microsatellite diversity

After the Sequential Goodness of Fit correction, deviation from HWE was occasional, likely arising from type 1 error, and therefore no locus was removed from the analyses (Table S3). No significant LD was found between any pair of loci (all $P > 0.05$).

Microsatellite diversity was moderate to high. *Ar* and H_E were positively correlated (r = 0.787, $P < 0.001$, n = 17). Hereafter we use *Ar* as our measure of microsatellite diversity. *Ar* was standardized for 22 individuals and ranged from 5.37 (Porto União) to 9.45 (Resende)

(Table 1). *Ar* was significantly different only between Porto União and Resende ($U = 93$, $P = 0.033$) and Porto União and Teresópolis ($U = 29$, $P = 0.039$).

There was a negative but non-significant correlation between *Ar* and size of the sampled area ($r = -0.114$, $P = 0.662$, $n = 17$) and between *Ar* and median elevation ($r = -0.084$, $P = 0.748$, $n = 17$).

Six populations had inbreeding coefficients (F_{IS}) significantly different from zero ($P < 0.05$). The highest F_{IS} (0.2177) was found in São José (Table 1).

Microsatellite differentiation

Global D_{est} was high (0.375, $P < 0.001$) and indicates population structure. Pairwise comparisons also detected population structure, since most θ values were between 0.05 and 0.15 (Table S4) and most D_{est} values were higher than 0.25 (Table 3). Pairwise θ and D_{est} were positively correlated ($r = 0.977$, $P < 0.001$, $n = 136$) and we use D_{est} as our measure of microsatellite differentiation hereafter. D_{est} ranged from 0.0204 (Guaratuba × Blumenau) to 0.8464 (Prudentópolis × Foz do Iguaçu). D_{est} high values were always detected in comparisons encompassing Porto União/Foz do Iguaçu and others. Low differentiation was observed in some populations near the coast (Iguape, Apiaí, Guaratuba, Blumenau, and São José) but also inland (Porto União × Foz do Iguaçu and Prudentópolis × Teodoro Sampaio).

Population structure was also suggested by the spatial cluster approach used by BAPS, which determined $K = 10$ as the most likely optimal number of clusters (probability of 98.99%). The clusters were [Foz do Iguaçu/Porto União], [Iguape/Apiaí/Guaratuba/ Blumenau/SãoJosé], [Ilha Comprida], [São Sebastião], [Ilhabela], [Ilha Grande], [Passa Quatro], [Teodoro Sampaio/Prudentópolis], [Teresópolis], [Resende/Angra dos Reis] (Figure 1). D_{est} results are in good agreement with these clusters.

229 The results of the migration rates estimated in BAYESASS suggested a low level of gene
230 flow throughout the studied area (Table S5). Only 15 out of 272 comparisons presented $m > 0$
231 between two populations. Most of the populations that presented gene flow are near the coast
232 (Figure 1), but populations inland such as Porto União × Foz do Iguaçu also showed evidence
233 of gene flow. Migration asymmetry was also detected. For instance, the non-differentiation
234 detected previously between Prudentópolis × Teodoro Sampaio is due to high migration rate
235 (0.2486) from Prudentópolis to Teodoro Sampaio, since the opposite was not detected (Table
236 S5). The results obtained by BAYESASS are in good agreement with the population structure
237 indicated by θ, D_{est} and BAPS.
238
239 *Isolation by distance*
240 There was a positive and significant correlation between geographic and genetic distance for
241 both mitochondrial ($r = 0.415$, $P = 0.004$, $n = 136$) and microsatellite markers ($r = 0.464$, $P <$
242 0.001, $n = 136$).
243
244 *Population demography*
245 The Wilcoxon sign test did not reveal recent bottleneck in any of the 17 populations (all $P >$
246 0.1392, Table S6). The model-shift test showed that the allele frequency distribution in all
247 populations show the expected L-shaped curve that is expected in the absence of recent
248 bottlenecks.

Discussion

Our results showed high structure in *T. angustula* populations. Populations were highly differentiated as demonstrated by mtDNA and microsatellite markers, suggesting that both females and males are low dispersers.

The mtDNA nucleotide diversity (π) ranged from low to high. High π suggests a long evolutionary history for the populations. Low π may be explained by lineage sorting or be an evidence of population bottleneck in the past. Several studies of population genetics and phylogeography of vertebrates and invertebrates, conducted in the same area we studied, have also found low mtDNA nucleotide diversity (Cabanne *et al.* 2007; Carnaval *et al.* 2009; Batalha-Filho *et al.* 2010; Brito & Arias 2010; Francisco & Arias 2010; D'Horta *et al.* 2011; Bell *et al.* 2012). As argued in these studies, during Pleistocene this geographic area seems to have witnessed ancient bottlenecks followed by species expansion.

For *T. angustula* a strong evidence of this phenomenon came from the haplotype network in star shape, centered in some haplotypes. According to Avise (2000) this shape is an indicative of bottleneck followed by population expansion. Therefore, it is likely that populations that present high mtDNA diversity (e.g. Angra dos Reis) remained in putative stable areas while populations with low mtDNA diversity (e.g. Passa Quatro) are in regions that likely used to be outside the refuges and may be result of the expansion of stable populations.

We found overall high mitochondrial genetic differentiation among populations. In despite of the species present wide distribution its populations are not homogenous. Similar population structure has been observed for other stingless bee species (Brito & Arias 2010; Francisco & Arias 2010; Quezada-Euán *et al.* 2012; Brito *et al.* 2013; Francisco *et al.* 2013). The mtDNA population structure seems to be reflecting the reproductive behavior of *T. angustula*. However, we verified that some populations are not differentiated from others.

This is likely due to gene flow but we cannot rule out the human role in mediating nests introduction in different areas. For instance, haplotypes 34, 35, and 36 all found in Ilha Comprida, were similar to those found in Passa Quatro/Teodoro Sampaio (34 and 36) and Teresópolis (35) (Figure S1). Due to the high rate of exclusive haplotypes and the distance among these populations, we believe these nests were transported to Ilha Comprida and caused an artificial increase in this population's mtDNA diversity.

Nuclear genetic diversity was moderate to high in all populations. The difference between genetic diversity obtained from mtDNA and microsatellites may indicate that the bottleneck that reduced mtDNA diversity in some populations might also have reduced nuclear diversity. However, microsatellite diversity would have increased since then due to its higher mutation rate (Estoup *et al.* 1996). Microsatellite diversity was not significantly different among all populations but Porto União. This result suggests that ecological features of each sampling site are not influencing the molecular diversity. Indeed, two environmental variables such as size of the sampled area and median elevation were not significantly correlated to the genetic diversity observed for both mtDNA and microsatellite markers.

High and significant inbreeding (F_{IS}) was found only in two populations (Foz do Iguaçu and São José). Nonetheless, these data should not be under deep concern since these two populations present high microsatellite diversity and are not genetically isolated. Moreover, our results did not detect recent bottleneck (e.g. due to habitat fragmentation) in any of the studied populations.

Microsatellite data also indicated high genetic structure and low gene flow among populations, suggesting that males also do not disperse over long distances even between populations separated by 34 km of continuous Atlantic Forest. It is interesting here to emphasize that all island populations were differentiated from their mainland counterparts, indicating that males cannot cross even 300 m or greater distances over water. The program

299 BAYESASS suggested that the highest migration rate was from Prudentópolis to Teodoro
300 Sampaio, populations separated by more than 300 km. These two populations do not share
301 any mtDNA haplotypes suggesting that this gene flow is mediated only by males across
302 generations.

303 For both markers population clusters appear to be unrelated to physical barriers (such
304 as rivers or mountain ranges) or forest presence, indicating that genetic connectivity demands
305 more than only habitat connectivity (Marsden *et al.* 2012). Populations may diverge even with
306 no obstacles to gene flow due to low dispersal, geographic distance and genetic drift (isolation
307 by distance). In a general view, population structure of *T. angustula* is shaped by isolation by
308 distance.

309 The highest divergence found for both markers was between Porto União/Foz do
310 Iguaçu and the remaining populations. At least 15 mtDNA mutation steps separate these two
311 populations from the others. This represents about 2.8 to 3.3% divergence, which is as high as
312 the divergence between lineages A and Y of *Apis mellifera* (Franck *et al.* 2001). Francisco *et*
313 *al.* (2014) suggested that bees from Porto União and Foz do Iguaçu might belong to the
314 subspecies *T. angustula fiebrigi* while the others to *T. angustula angustula*.

315 Among the islands we visited only Ilha do Mel (Zanella 2005), Ilha de Santa Catarina
316 (Steiner *et al.* 2006) and Ilha Grande (Lorenzon *et al.* 2006) had been previously surveyed for
317 bees and *T. angustula* was reported on all of them. We did not locate *T. angustula* on six of
318 the 11 islands visited. The failure in collecting *T. angustula* in most islands may be due its
319 ancestral absence in the islands when they were isolated or due to its extinction after isolation.
320 The constraint on queen dispersal prevents (re)colonization of islands whose distance from
321 the mainland is greater than a few hundred of meters. Even if (re)colonization has occurred,
322 its settlement may not have been successful. With low dispersal, *T. angustula* has low
323 effective population size and high extinction rate. Island size may be critical to the survival of

viable *T. angustula* populations – we were unable to locate them on any island less than 28 km^2. Competition among colonies doubtless limits the number of colonies an island can support so that small islands may not be able to maintain viable populations of *T. angustula*. The rarity of stingless bee species on islands has been noted elsewhere (Schwartz-Filho & Laroca 1999; Zanella 2005).

Our results indicate that *T. angustula* is not genetically homogeneous across the studied area. Considering that this species has a continental distribution, we speculate this species is very old and probably constituted by a wide range of genetically different taxa with the same (or similar) morphology. Sampling across its entire distribution range is needed to elucidate its taxonomic status as well as its evolutionary history.

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Table 1. Population characteristics and genetic diversity in *Tetragonisca angustula* populations. SA: sampled area in square kilometers. ME: median elevation in meters. N: sample size. NH: number of haplotypes. h ± sd: haplotype diversity and standard deviation. π ± sd: nucleotide diversity and standard deviation. Ar ± se: allelic richness after rarefaction for 22 individuals and standard error. H_E ± se: expected heterozigosity and standard error. F_{IS}: inbreeding coefficient.

Population	SA (km^2)	ME (m)	N	NH	h ± sd	π ± sd	Ar ± se	H_E ± se	F_{IS}
1. Teresópolis (TERE)	253	939	46	5	0.207 ± 0.079	0.0087 ± 0.0080	9.07 ± 1.27	0.770 ± 0.045	0.0251
2. Resende (RESE)	10	444	44	3	0.394 ± 0.072	0.0065 ± 0.0066	9.45 ± 1.40	0.773 ± 0.054	0.0248
3. Passa Quatro (PASQ)	385	911	48	2	0.042 ± 0.039	0.0006 ± 0.0019	6.02 ± 0.97	0.626 ± 0.082	-0.0185
4. Angra dos Reis (ANGR)	1208	12	57	7	0.642 ± 0.049	0.0369 ± 0.0225	8.97 ± 1.47	0.769 ± 0.041	0.0463*
5. Ilha Grande (IGRA)	193[&]	11	57	5	0.687 ± 0.032	0.0153 ± 0.0116	6.50 ± 1.26	0.682 ± 0.057	0.0247
6. São Sebastião (SSEB)	117	14	46	3	0.478 ± 0.052	0.0075 ± 0.0072	8.05 ± 1.32	0.712 ± 0.050	0.0088
7. Ilha de São Sebastião (IBEL)	336[&]	25	49	3	0.190 ± 0.072	0.0036 ± 0.0047	6.11 ± 0.98	0.636 ± 0.061	0.0062
8. Iguape (GUAP)	67	13	38	3	0.152 ± 0.077	0.0070 ± 0.0070	6.75 ± 1.13	0.637 ± 0.080	-0.0353
9. Ilha Comprida (ICOM)	200[&]	10	22	4	0.398 ± 0.122	0.0407 ± 0.0251	5.64 ± 0.69	0.659 ± 0.054	0.0381
10. Apiaí (APIA)	504	161	45	6	0.625 ± 0.068	0.0152 ± 0.0115	7.35 ± 1.40	0.634 ± 0.086	0.0142
11. Guaratuba (GUAR)	341	15	36	6	0.636 ± 0.061	0.0233 ± 0.0159	7.02 ± 1.28	0.611 ± 0.084	0.038
12. Blumenau (BLUM)	389	36	45	8	0.628 ± 0.062	0.0127 ± 0.0102	7.38 ± 1.48	0.594 ± 0.102	0.0857**
13. São José (SJOS)	841	11	24	5	0.493 ± 0.116	0.0084 ± 0.0080	7.29 ± 1.48	0.604 ± 0.092	0.2177**
14. Prudentópolis (PRUD)	885	811	42	5	0.302 ± 0.089	0.0049 ± 0.0056	6.35 ± 0.97	0.621 ± 0.070	0.0404
15. Porto União (PUNI)	1521	913	35	5	0.506 ± 0.090	0.0086 ± 0.0080	5.37 ± 0.94	0.513 ± 0.072	0.0832**
16. Foz do Iguaçu (FOZI)	5148	543	43	9	0.632 ± 0.074	0.0157 ± 0.0119	6.59 ± 1.15	0.555 ± 0.064	0.1389**
17. Teodoro Sampaio (TSAM)	5550	440	45	9	0.716 ± 0.058	0.0179 ± 0.0130	7.22 ± 1.13	0.616 ± 0.081	0.0965**

[&]: island area; *: $P < 0.05$; **: $P < 0.01$.

Table 2. Estimates of evolutionary divergence over sequence pairs between populations of *Tetragonisca angustula*. The number of base substitutions per site from averaging over all sequence pairs between populations are shown. Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980) and involved 722 nucleotide sequences. Population abbreviations as in Table 1.

	TERE	RESE	PASQ	ANGR	IGRA	SSEB	IBEL	GUAP	ICOM	APIA	GUAR	BLUM	SJOS	PRUD	PUNI	FOZI
RESE	0.0071															
PASQ	0.0085	0.0109														
ANGR	0.0077	0.0101	0.0067													
IGRA	0.0079	0.0103	0.0069	0.0042												
SSEB	0.0094	0.0118	0.0083	0.0064	0.0076											
IBEL	0.0084	0.0108	0.0074	0.0056	0.0066	0.0010										
GUAP	0.0088	0.0111	0.0077	0.0058	0.0070	0.0012	0.0004									
ICOM	0.0082	0.0111	0.0067	0.0062	0.0072	0.0028	0.0020	0.0000								
APIA	0.0084	0.0107	0.0078	0.0057	0.0073	0.0016	0.0008	0.0022	0.0025							
GUAR	0.0089	0.0113	0.0073	0.0061	0.0072	0.0020	0.0012	0.0014	0.0028	0.0017						
BLUM	0.0083	0.0111	0.0078	0.0060	0.0071	0.0015	0.0007	0.0009	0.0024	0.0013	0.0017					
SJOS	0.0089	0.0113	0.0079	0.0059	0.0071	0.0012	0.0005	0.0007	0.0023	0.0011	0.0015	0.0010				
PRUD	0.0099	0.0123	0.0015	0.0081	0.0083	0.0097	0.0088	0.0090	0.0081	0.0092	0.0087	0.0092	0.0092			
PUNI	0.0319	0.0317	0.0295	0.0315	0.0339	0.0329	0.0319	0.0319	0.0321	0.0317	0.0314	0.0323	0.0323	0.0308		
FOZI	0.0311	0.0309	0.0287	0.0308	0.0331	0.0321	0.0312	0.0312	0.0313	0.0310	0.0306	0.0315	0.0315	0.0301	0.0012	
TSAM	0.0088	0.0111	0.0009	0.0070	0.0072	0.0085	0.0076	0.0079	0.0071	0.0081	0.0076	0.0081	0.0081	0.0023	0.0297	0.0289

Table 3. Pairwise index of differentiation (D_{est}) from microsatellite data of *Tetragonisca angustula*. Population abbreviations as in Table 1.

	TERE	RESE	PASQ	ANGR	IGRA	SSEB	IBEL	GUAP	ICOM	APIA	GUAR	BLUM	SJOS	PRUD	PUNI	FOZI
RESE	0.1074															
PASQ	0.2821	0.2177														
ANGR	0.1761	0.0988	0.2842													
IGRA	0.3183	0.2389	0.4053	0.1735												
SSEB	0.2866	0.2082	0.4191	0.1710	0.1186											
IBEL	0.4341	0.3806	0.4261	0.3119	0.2912	0.2144										
GUAP	0.3914	0.3053	0.4394	0.2455	0.1733	0.0975	0.2064									
ICOM	0.3129	0.2739	0.3395	0.2152	0.1883	0.1594	0.2254	0.0992								
APIA	0.3826	0.3016	0.4209	0.2389	0.1904	0.1042	0.1802	0.0300	0.1356							
GUAR	0.3787	0.3153	0.4012	0.2914	0.2115	0.1251	0.2069	0.0912	0.1568	0.0445						
BLUM	0.3866	0.3101	0.4063	0.2943	0.1854	0.1298	0.2300	0.0551	0.1550	0.0328	0.0204					
SJOS	0.3928	0.3339	0.4518	0.3108	0.2159	0.1234	0.1959	0.0834	0.2113	0.0468	0.0375	0.0212				
PRUD	0.2772	0.2424	0.1387	0.3213	0.4331	0.4518	0.5061	0.4647	0.3608	0.4803	0.4253	0.4341	0.4854			
PUNI	0.7951	0.7547	0.8380	0.6959	0.7074	0.6485	0.6971	0.6693	0.7351	0.6764	0.7051	0.6824	0.6344	0.8281		
FOZI	0.7863	0.7375	0.8439	0.6896	0.6589	0.5886	0.6663	0.6296	0.7007	0.6154	0.6276	0.6061	0.5685	0.8464	0.0274	
TSAM	0.2649	0.2257	0.1173	0.3242	0.3981	0.4218	0.4545	0.4396	0.3552	0.4504	0.4043	0.4052	0.4466	0.0308	0.8015	0.8169

Colours highlight D_{est} values. Green: $D_{est} < 0.05$; yellow: $0.05 < D_{est} < 0.15$; orange: $0.15 < D_{est} < 0.25$; red: $D_{est} > 0.25$. All $P < 0.0054$.

Figure legend

Fig. 1. Posterior probability assignment (vertical axis) of individual genotypes (horizontal axis) for $K = 10$ (*Tetragonisca angustula*) according to the program BAPS (upper panel). Below, map of the studied area with the approximate location of the sampled populations. Population names are 1: Teresópolis, 2: Resende, 3: Passa Quatro, 4: Angra dos Reis, 5: Ilha Grande, 6: São Sebastião, 7: Ilha de São Sebastião, 8: Iguape, 9: Ilha Comprida, 10: Apiaí, 11: Guaratuba, 12: Blumenau, 13: São José, 14: Prudentópolis, 15: Porto União, 16: Foz do Iguaçu, and 17: Teodoro Sampaio. Detailed location of all islands visited (lower panels). IGRA: Ilha Grande; IANC: Ilha Anchieta; ITMD: Ilha do Tamanduá; IVIT: Ilha da Vitória; IBUZ: Ilha de Búzios; IBEL: Ilha de São Sebastião; IMTG: Ilha Monte de Trigo. ICOM: Ilha Comprida; ICAR: Ilha do Cardoso; IMEL: Ilha do Mel. ISCA: Ilha de Santa Catarina.