

1 **Genetic structure of the stingless bee *Tetragonisca angustula***

2 Flávio O. Francisco^{1,2}, Leandro R. Santiago¹, Yuri M. Mizusawa¹, Benjamin P. Oldroyd² and

3 Maria C. Arias¹

4

5 ¹*Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de*

6 *São Paulo, Rua do Matão 277 – sala 320, São Paulo, SP 05508-090, Brazil*

7 ²*Behaviour and Genetics of Social Insects Lab, School of Biological Sciences A12, University*

8 *of Sydney, Sydney, NSW 2006, Australia*

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11 Correspondence:

12 Flávio O. Francisco, Departamento de Genética e Biologia Evolutiva, Instituto de Biociências,

13 Universidade de São Paulo, Rua do Matão 277 – sala 320, 05508-090, São Paulo, SP, Brazil.

14 Tel: +55 11 30917587; Fax: +55 11 30917553; email: fofrancisco@usp.br

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16 Running title: Genetic structure of *T. angustula*

17

18 **Abstract**

19 The stingless bee *Tetragonisca angustula* Latreille 1811 is distributed from Mexico to
20 Argentina and is one of the most widespread bee species in the Neotropics. However, this
21 wide distribution contrasts with the short distance traveled by females to build new nests.
22 Here we evaluate the genetic structure of several populations of *T. angustula* using
23 mitochondrial DNA and microsatellites. These markers can help us to detect differences in the
24 migratory behavior of males and females. Our results show that the populations are highly
25 differentiated suggesting that both females and males have low dispersal distance. Therefore,
26 its continental distribution probably consists of several cryptic species.

27

28 **Key words** islands; Meliponini; microsatellites; mtDNA; population genetics

29 **Introduction**

30 The stingless bee *Tetragonisca angustula* Latreille 1811 distributed from Mexico to Argentina
31 is one of the most widespread bee species in the Neotropics (Silveira *et al.*, 2002; Camargo &
32 Pedro, 2013). It is a small (4-5 mm in length), generalist and highly eusocial bee (Michener,
33 2007) and highly adaptable to different nest sites. Colonies comprise up to 5,000 individuals
34 (Lindauer & Kerr, 1960), and are usually built in tree trunks or in wall cavities. It swarms
35 frequently and is extremely successful in urban environments (Batista *et al.*, 2003; Slaa, 2006;
36 Velez-Ruiz *et al.*, 2013). *Tetragonisca angustula* is one of the most popular stingless bees for
37 meliponiculture in Latin America (Nogueira-Neto, 1997; Cortopassi-Laurino *et al.*, 2006) and
38 nest transportation and trading is very common among beekeepers.

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

49 In contrast, little is known about the reproductive behavior of stingless bee males.
50 After emergence from brood cells, they remain in the nest for two to three weeks (Cortopassi-
51 Laurino, 2007). They then leave the nest and never return. There are no data about the
52 behavior of males during their period outside the nest. In the laboratory, males can live up to
53 six weeks (Velthuis *et al.*, 2005). Therefore, they likely have two to four weeks for dispersal

54 and reproduction. Males often form mating aggregations, and these are comprised of males
55 from hundreds of different, and not necessarily nearby colonies (Paxton, 2000; Cameron *et*
56 *al.*, 2004; Kraus *et al.*, 2008; Mueller *et al.*, 2012). This suggests high male dispersal.

57 Studies on the genetic structure of populations can help us better understand dispersal
58 behavior and evolutionary history. There are three existing population genetic studies of *T.*
59 *angustula* (Oliveira *et al.*, 2004; Baitala *et al.*, 2006; Stuchi *et al.*, 2008). These studies are of
60 limited scope due to limited sampling or the use of outdated molecular markers such as
61 RAPDs and isozymes. Thus, the conclusions are ambiguous concerning the extent and
62 direction of gene flow, population differentiation, dispersal and evolutionary history.

63 Here we evaluate the genetic structure of several populations of *T. angustula* using
64 mitochondrial DNA (mtDNA) and microsatellite markers. Considering the wide distribution
65 of *T. angustula* and the commonness of nest transportation and trading, we expect low genetic
66 differentiation among populations despite the low dispersal distance of females during
67 swarming.

68

69

70 **Materials and methods**

71 *Sampling*

72 We collected 1,002 *T. angustula* from 457 sites distributed on the mainland and islands in
73 south/south-eastern Brazil (Table S1). Eleven islands all with arboreal vegetation and of area
74 greater than 1.0 km² were selected, 10 being land-bridge islands isolated about 12,000 years
75 ago (Suguió *et al.*, 2005) and one sedimentary island (Ilha Comprida) which arose about
76 5,000 years ago (Suguió *et al.*, 2003). The islands range in size from 1.1 to 451 km² and are
77 0.1 to 38 km from the mainland (Table S2, Fig. 1). Bees were sampled from nests ($n = 125$,

78 one per nest) and flowers ($n = 877$) (Table S1). Samples were grouped into 17 populations, 14
79 from the mainland and three from islands (Fig. 1).

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

83

84 *Mitochondrial DNA sequencing*

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

88

89 *Microsatellite genotyping*

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

94 MICRO-CHECKER 2.2.3 (van Oosterhout *et al.*, 2004) was used to identify null alleles
95 and scoring errors. COLONY 2.0.1.7 (Jones & Wang, 2010) was used to determine whether
96 individuals collected in the same plant or places nearby were related. Samples were excluded
97 from our data set if matched all of the following three criteria: collected at sites distant less
98 than 2 km, indicated as related by COLONY, and sharing a mtDNA haplotype. Overall, 722 *T.*
99 *angustula* bees from 17 populations were deemed suitable for further genetic analyses (Table
100 1).

101 GENEPOP 4.1.2 (Rousset, 2008) was used to verify Hardy-Weinberg equilibrium
102 (HWE) in populations and loci and to detect linkage disequilibrium (LD). Markov chain was

103 set for 10,000 dememorizations, 1,000 batches and 10,000 iterations per batch. In cases of
104 multiple comparisons, P -values were corrected by applying Sequential Goodness of Fit test by
105 the program SGOF 7.2 (Carvajal-Rodríguez *et al.*, 2009). This method is advantageous over
106 other correction methods because it increases its statistical power with the increasing of the
107 number of tests (Carvajal-Rodríguez *et al.*, 2009).

108

109 *Genetic diversity*

110 ARLEQUIN 3.5.1.3 (Excoffier & Lischer, 2010) was used to calculate mtDNA haplotype (h)
111 and nucleotide (π) diversity. GENALEX 6.5 (Peakall & Smouse, 2006, 2012) was used to
112 calculate microsatellite allelic richness (A) and expected heterozygosity (H_E). Since sample
113 sizes were different, allelic richness was standardized by rarefaction (A_r) using the program
114 HP-RARE 1.0 (Kalinowski, 2005). Differences in A_r among populations were estimated by
115 Mann-Whitney two-tailed U Test (Mann & Whitney, 1947). Inbreeding coefficients (F_{IS})
116 were calculated for each population with 10,000 permutations using ARLEQUIN.

117

118 *Population differentiation and gene flow*

119 MEGA 5.2.1 (Tamura *et al.*, 2011) was used to calculate the number of base substitutions per
120 site from mitochondrial sequences by averaging over all sequence pairs between populations
121 using the Kimura 2-parameter (K2p) model (Kimura, 1980). Population pairwise θ values (an
122 F_{ST} analogue, Weir & Cockerham 1984) were calculated with 10,000 permutations by
123 ARLEQUIN using microsatellite alleles. When heterozygosity is high, F_{ST} and its analogues
124 may not be appropriate measures of genetic differentiation (Hedrick, 2005; Jost, 2008; Heller
125 & Siegismund, 2009). For this reason, Jost's D_{est} (Jost, 2008) was calculated. This statistic is
126 not influenced by heterozygosity (Jost, 2008) and is more appropriate for microsatellite data
127 (Heller & Siegismund, 2009). Global D_{est} was calculated with 9,999 permutations for mtDNA

128 and microsatellite data using GENALEX. Pairwise D_{est} was calculated only for microsatellite
129 data. Mantel tests between genetic and geographical distances among populations were
130 performed with 9999 permutations by GENALEX to verify isolation by distance for both
131 molecular markers.

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

140 Estimates of rates and direction of current and/or recent migration (m) between
141 populations were determined by the program BAYESASS 3 (Wilson & Rannala, 2003) using
142 microsatellites multilocus genotypes through Markov chain Monte Carlo (MCMC)
143 techniques. We performed five independent runs with 10^7 MCMC iterations, burn-in of 10^6
144 iterations and sampling frequency of 2,000. The delta values used were 0.25 (migration), 0.40
145 (allele frequencies) and 0.55 (inbreeding).

146

147 *Assessment of population demography*

148 To detect any recent bottleneck events we used the program BOTTLENECK 1.2.02 (Piry *et al.*,
149 1999). We used the two-phased model (TPM) of mutation which is suggested as the most
150 appropriate for microsatellites (Di Rienzo *et al.*, 1994). The variance among multiple steps
151 was 12 and the proportion of stepwise mutation model in the TPM was 95% as suggested by

152 Piry *et al.* (1999). Altogether 10,000 iterations were performed. The significance of any
153 deviation was determined with a Wilcoxon sign-rank test.

154

155

156 **Results**

157 *Island occurrences*

158 *Tetragonisca angustula* was found and collected on five of the 11 islands visited (Table 1).

159 However, only the samples from Ilha Grande, Ilha de São Sebastião and Ilha Comprida were
160 included in the analyses. The other collections were not included due to small sample size
161 (Ilha do Cardoso, $n = 1$), and to individuals being highly related, with anecdotal reports of
162 introduced nests (Ilha de Santa Catarina, see Francisco *et al.* (2014)).

163

164 *MtDNA diversity*

165 The *COI* gene sequences were 417 bp long (GenBank accession numbers KF222891-
166 KF223893) and 32 haplotypes were identified. The *Cytb* sequences were 391 bp long
167 (KF223894-KF224896) and generated 43 haplotypes. Most differences among haplotypes
168 were synonymous substitutions, since the number of distinct amino acid sequences were four
169 for *COI* and 15 for *Cytb*. We concatenated the nucleotide sequences (808 bp) for population
170 analyses.

171 The 722 concatenated sequences defined 73 haplotypes. Since h and π were positively
172 correlated ($r = 0.510$, $P = 0.036$, $n = 17$) we hereafter use π as our measure of mtDNA
173 diversity. Nucleotide diversity ranged from 0.0006 ± 0.0019 (Passa Quatro) to $0.0407 \pm$
174 0.0251 (Ilha Comprida) (Table 1).

175 There was a non-significant positive correlation between the size of the sampled area
176 and mtDNA diversity ($r = 0.135$, $P = 0.606$, $n = 17$). The correlation between median

177 elevation and mtDNA diversity was negative but non-significant ($r = -0.428$, $P = 0.087$, $n =$
178 17).

179

180 *MtDNA differentiation*

181 Population structure was high. Of 73 haplotypes 67 were population-specific. We built a
182 haplotype network where the frequency and distribution of haplotypes are shown (Fig. S1).
183 The network shows a ‘star-pattern’ centered on four haplotypes. It illustrates the high number
184 of endemic haplotypes, and the great number of nucleotide substitutions that separate the
185 Porto União/Foz do Iguaçu populations from the others. The populations of Teresópolis,
186 Resende, Prudentópolis, Angra dos Reis, and Ilha Grande all feature unique haplotypes.

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

190

191 *Microsatellite diversity*

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

195 Microsatellite diversity was moderate to high. A_r and H_E were positively correlated (r
196 = 0.787, $P < 0.001$, $n = 17$). Hereafter we use A_r as our measure of microsatellite diversity. A_r
197 was standardized for 22 individuals and ranged from 5.37 (Porto União) to 9.45 (Resende)
198 (Table 1). A_r was significantly different only between Porto União and Resende ($U = 93$, $P =$
199 0.033) and Porto União and Teresópolis ($U = 29$, $P = 0.039$).

200 There was a negative but non-significant correlation between A_r and size of the
201 sampled area ($r = -0.114$, $P = 0.662$, $n = 17$) and between A_r and median elevation ($r = -$

202 0.084, $P = 0.748$, $n = 17$).

203 Six populations had inbreeding coefficients (F_{IS}) significantly different from zero ($P <$
204 0.05). The highest F_{IS} (0.2177) was found in São José (Table 1).

205

206 *Microsatellite differentiation*

207 Global D_{est} was high (0.375, $P < 0.001$) and indicates population structure. Pairwise
208 comparisons also detected population structure, since most θ values were between 0.05 and
209 0.15 (Table S4) and most D_{est} values were higher than 0.25 (Table 3). Pairwise θ and D_{est}
210 were positively correlated ($r = 0.977$, $P < 0.001$, $n = 136$) and we use D_{est} as our measure of
211 microsatellite differentiation hereafter. D_{est} ranged from 0.0204 (Guaratuba \times Blumenau) to
212 0.8464 (Prudentópolis \times Foz do Iguaçu). High D_{est} values were always detected in
213 comparisons between Porto União/Foz do Iguaçu and other populations. Low differentiation
214 was observed in some populations near the coast (Iguape, Apiaí, Guaratuba, Blumenau, and
215 São José) but also inland (Porto União \times Foz do Iguaçu and Prudentópolis \times Teodoro
216 Sampaio).

217 Population structure was also suggested by the spatial cluster approach used by BAPS,
218 which determined $K = 10$ as the most likely optimal number of clusters (probability of
219 98.99%). The clusters were [Foz do Iguaçu/Porto União], [Iguape/Apiaí/Guaratuba/
220 Blumenau/São José], [Ilha Comprida], [São Sebastião], [Ilhabela], [Ilha Grande], [Passa
221 Quatro], [Teodoro Sampaio/Prudentópolis], [Teresópolis], [Resende/Angra dos Reis] (Fig. 1).
222 D_{est} results are in good agreement with these clusters.

223 The results of the migration rates estimated in BAYESASS suggested a low level of gene
224 flow throughout the studied area (Table S5). Only 15 out of 272 comparisons showed $m > 0$
225 between two populations. Most of the populations that showed evidence of gene flow are near
226 the coast (Fig. 1), but inland populations such as Porto União \times Foz do Iguaçu also showed

227 evidence of gene flow. Migration is directional. For instance, the non-differentiation detected
228 between Prudentópolis × Teodoro Sampaio is apparently due to a high migration rate (0.2486
229 migrants per generation) from Prudentópolis to Teodoro Sampaio, whereas migration in the
230 opposite direction was not detected (Table S5). The results obtained by BAYESASS are in good
231 agreement with the population structure indicated by θ , D_{est} and BAPS.

232

233 *Isolation by distance*

234 There was a positive and significant correlation between geographic and genetic distance for
235 both mitochondrial ($r = 0.415$, $P = 0.004$, $n = 136$) and microsatellite markers ($r = 0.464$, $P <$
236 0.001 , $n = 136$).

237

238 *Population demography*

239 We did not detect recent bottlenecks in any of the 17 populations (all $P > 0.1392$, Table S6).

240

241 **Discussion**

242 Our results show that *T. angustula* populations are highly differentiated as demonstrated by
243 mtDNA and microsatellite markers. This suggests that both females and males have low
244 dispersal distance.

245 Per population mtDNA nucleotide diversity (π) ranged from low to high. High π
246 suggests that a population had a long evolutionary history and a large effective population
247 size. Low π may be explained by lineage sorting or suggest that a population bottleneck has
248 occurred in the past (Avice, 2000). The characteristic star shape of the *T. angustula* haplotype
249 network provides evidence of relatively recent local extinction, re-colonization, and
250 population expansion. Several phylogeographic studies of vertebrate and invertebrate
251 populations, conducted in some of the areas that we studied, also found low mtDNA
252 nucleotide diversity (Cabanne *et al.*, 2007; Carnaval *et al.*, 2009; Batalha-Filho *et al.*, 2010;
253 Brito & Arias, 2010; Francisco & Arias, 2010; D'Horta *et al.*, 2011; Bell *et al.*, 2012). As
254 argued in these papers, changes in sea level during the Pleistocene generated population
255 bottlenecks followed by species expansion, and this is reflected in localized low nucleotide
256 diversity to this day. Therefore, it is likely that populations that have high mtDNA diversity
257 (e.g. Angra dos Reis) did not experience recent bottlenecks, while populations with low
258 mtDNA diversity (e.g. Passa Quatro) are in regions that likely arose by a recent population
259 expansion.

260 Overall, we found high mitochondrial genetic differentiation between populations.
261 Similar population structuring has been observed for other stingless bee species (Brito &
262 Arias, 2010; Francisco & Arias, 2010; Quezada-Euán *et al.*, 2012; Brito *et al.*, 2013;
263 Francisco *et al.*, 2013). The mtDNA population structure of stingless bees probably arises
264 from their reproductive behavior. Nonetheless, some populations are not well differentiated

265 from others. This is likely due to gene natural flow, although human transportation also likely
266 plays a role. For instance, haplotypes 34, 35, and 36 all found in Ilha Comprida, were similar
267 to those found in Passa Quatro/Teodoro Sampaio (34 and 36) and Teresópolis (35) (Fig. S1).
268 Due to the high frequency of endemic haplotypes, and the physical distance between these
269 populations, we suggest that nests have been transported to Ilha Comprida causing an
270 artificial increase in this population's mtDNA diversity.

271 Nuclear genetic diversity was moderate to high in all populations. Microsatellite
272 diversity was not significantly different between populations except for Porto União. This
273 result shows that the ecological features of each sampling site are not influencing the
274 molecular diversity. Indeed, variables such as size of the sampled area and median elevation
275 were not significantly correlated with genetic diversity for both mtDNA and microsatellites.
276 Moreover, our results did not detect recent bottleneck (e.g. due to habitat fragmentation) in
277 any of the studied populations. However, it is worth emphasizing that theoretical and practical
278 studies have shown that habitat fragmentation affects immediately the genetic structure by
279 increasing it, while the reduction of genetic diversity may take longer (Varvio *et al.*, 1986;
280 Keyghobadi *et al.*, 2005). According to this statement, it will be necessary to monitor the
281 genetic diversity of *T. angustula* populations studied over years, since currently high structure
282 was detected.

283 Evidence of inbreeding was found in six populations (Angra dos Reis, Blumenau, São
284 José, Porto União, Foz do Iguaçu and Teodoro Sampaio). This might be an artifact caused by
285 Wahlund effect (Hartl & Clark, 2007) and/or a consequence of the low dispersal of *T.*
286 *angustula*. If the latter is true, the persistence of these populations is at risk (Keller & Waller,
287 2002).

288 Microsatellite data also indicated high genetic structuring and low gene flow among
289 populations. This suggests that like females, the dispersal distance of males is also quite

290 limited even between populations separated by 34 km of continuous forest. It is interesting
291 here to note that all island populations were differentiated from their mainland counterparts,
292 indicating that males do not cross water for distances as short as 300 m. The program
293 BAYESASS suggested that the highest migration rate is from Prudentópolis to Teodoro
294 Sampaio, populations separated by more than 300 km. These two populations do not share
295 any mtDNA haplotypes suggesting that this gene flow is mediated only by males following
296 the stepping stone model (Kimura & Weiss, 1964).

297 For both markers population clusters appear to be unrelated to physical barriers (such
298 as rivers or mountain ranges) or forest presence, indicating that genetic connectivity demands
299 more than just habitat connectivity (Marsden *et al.*, 2012). Populations may diverge even
300 when there are no apparent obstacles to gene flow due to low dispersal, geographic distance
301 and genetic drift (isolation by distance). Overall, the population structure of *T. angustula* is
302 shaped by isolation by distance.

303 The highest genetic divergence observed was between Porto União/Foz do Iguacu and
304 the remaining populations. At least 15 mtDNA mutation steps separate these two populations
305 from the others. This represents about 2.8 to 3.3% divergence, which is as high as the
306 divergence between lineages A and Y of *Apis mellifera* (Franck *et al.*, 2001), which are
307 thought to have diverged over one million years ago (Whitfield *et al.*, 2006). Francisco *et al.*
308 (2014) suggested that bees from Porto União and Foz do Iguacu might belong to the
309 subspecies *T. angustula fiebrigi* while the others to *T. angustula angustula*.

310 Among the islands we visited only Ilha do Mel (Zanella, 2005), Ilha de Santa Catarina
311 (Steiner *et al.*, 2006) and Ilha Grande (Lorenzon *et al.*, 2006) had been previously surveyed
312 for bees and *T. angustula* was reported on all of them. We did not locate *T. angustula* on six
313 of the 11 islands we visited. Our failure to verify *T. angustula* on most islands may be due its
314 ancestral absence on the islands when they became isolated or to its extinction after isolation.

315 The constraint on queen dispersal prevents (re)colonization of islands whose distance from
316 the mainland is greater than a few hundred of meters. Even if (re)colonization has occurred,
317 its establishment may not have been successful. With low dispersal, *T. angustula* has low
318 effective population size and high extinction rate. Island size may be critical to the survival of
319 viable *T. angustula* populations – we were unable to locate them on any island less than 28
320 km². Competition among colonies doubtless limits the number of colonies an island can
321 support so that small islands may not be able to maintain viable populations of *T. angustula*.
322 The rarity of stingless bee species on islands has been noted elsewhere (Schwartz-Filho &
323 Laroça, 1999; Zanella, 2005).

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

329

330

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348

349

350 **Disclosure**

351 The authors have no conflict of interest.

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- 544

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

| Population | SA (km ²) | ME (m) | N | NH | $h \pm sd$ | $\pi \pm sd$ | $Ar \pm se$ | $H_E \pm se$ | 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** |

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

550 **Table 2** Estimates of evolutionary divergence over sequence pairs between populations of *Tetragonisca angustula*. The number of base
 551 substitutions per site from averaging over all sequence pairs between populations are shown. Analyses were conducted using the Kimura 2-
 552 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 |

554

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 |

555

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

556

557 **Figure legend**

558

559 **Fig. 1** Posterior probability assignment (vertical axis) of individual genotypes (horizontal
560 axis) for $K = 10$ (*Tetragonisca angustula*) according to the program BAPS (upper panel).

561 Below, map of the studied area with the approximate location of the sampled populations.

562 Population names are 1: Teresópolis, 2: Resende, 3: Passa Quatro, 4: Angra dos Reis, 5: Ilha

563 Grande, 6: São Sebastião, 7: Ilha de São Sebastião, 8: Iguape, 9: Ilha Comprida, 10: Apiaí, 11:

564 Guaratuba, 12: Blumenau, 13: São José, 14: Prudentópolis, 15: Porto União, 16: Foz do

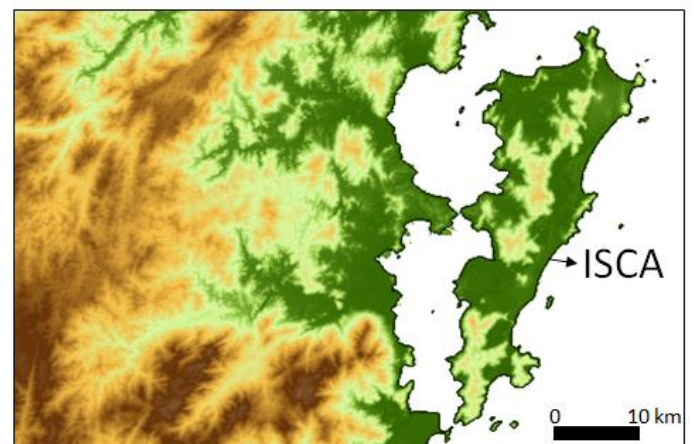
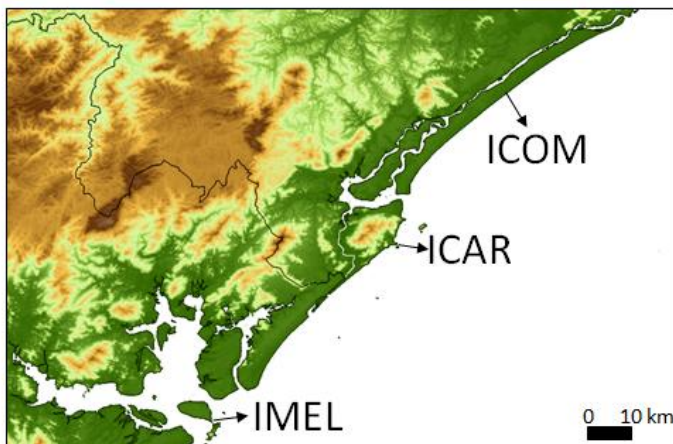
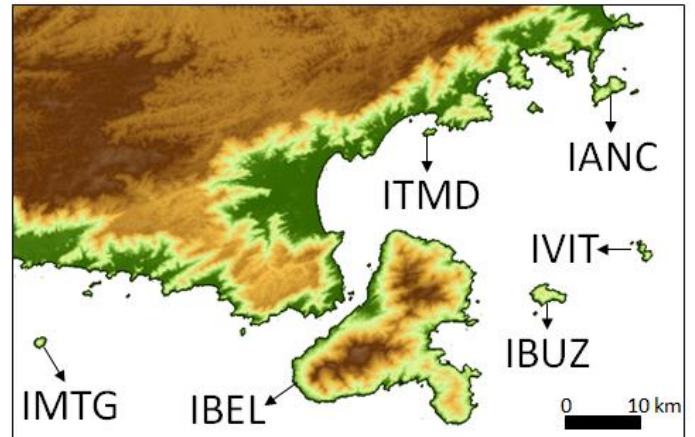
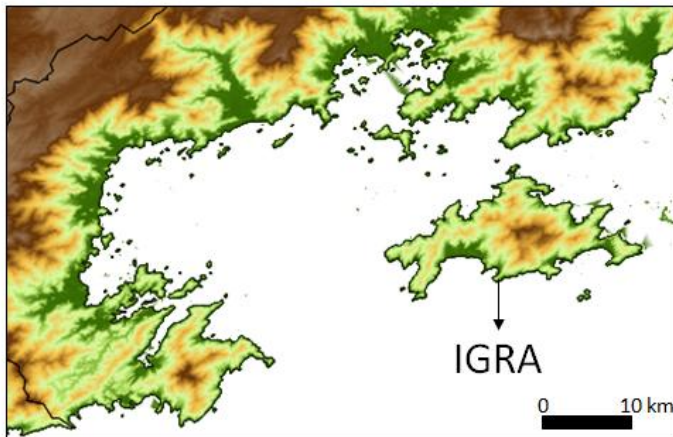
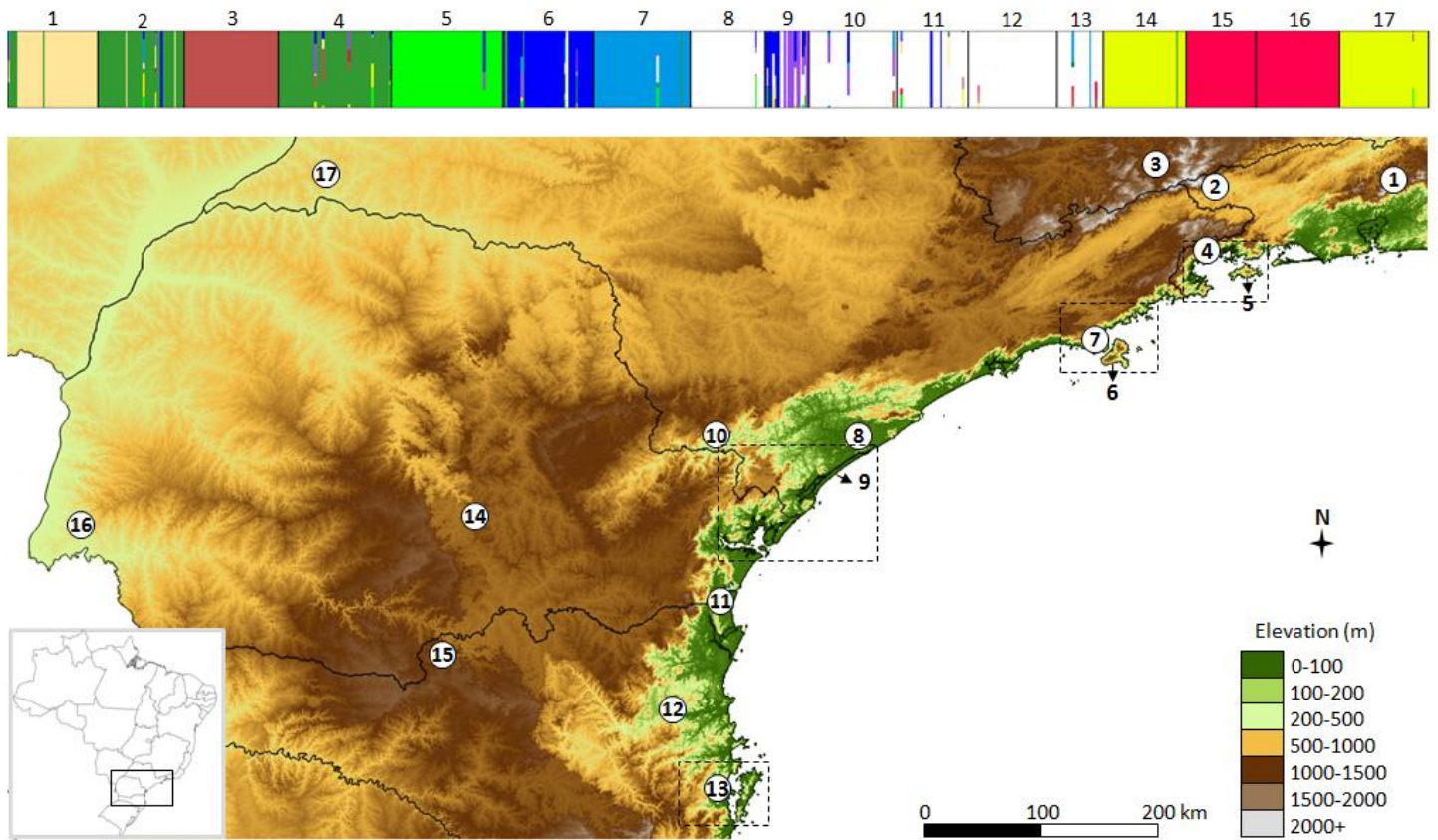
565 Iguaçú, and 17: Teodoro Sampaio. Detailed location of all islands visited (lower panels).

566 IGRA: Ilha Grande; IANC: Ilha Anchieta; ITMD: Ilha do Tamanduá; IVIT: Ilha da Vitória;

567 IBUZ: Ilha de Búzios; IBEL: Ilha de São Sebastião; IMTG: Ilha Monte de Trigo. ICOM: Ilha

568 Comprida; ICAR: Ilha do Cardoso; IMEL: Ilha do Mel. ISCA: Ilha de Santa Catarina.

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