

1 **Geometric morphometrics discriminates Eastern and Western populations of**
2 ***Partamona rustica* (Hymenoptera, Apidae, Meliponini) separated by São Francisco**
3 **River**

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5 Vinicius Oliveira e Silva¹, Tiago Maurício Franco², Elder Assis Miranda³, Cíntia Akemi
6 Oi⁴, Kátia Maria Ferreira¹, Marco Antonio Del Lama^{1*}

7

8 ¹ Laboratório de Genética Evolutiva de Himenópteros, Departamento de Genética e
9 Evolução, Universidade Federal de São Carlos, Rodovia Washington Luiz, km 235,
10 13565-905, São Carlos, São Paulo, Brazil

11 ² Escola de Artes, Ciências e Humanidades – EACH/USP. Rua Arlindo Bétio, 1000.
12 03828-000, São Paulo, São Paulo, Brazil.

13 ³ Curso Técnico em Meio Ambiente, Instituto Federal de Educação Ciência e
14 Tecnologia do Tocantins, Av. Tocantínia, 566 - Jardim América, 77500-000, Porto
15 Nacional - Tocantins, Brazil.

16 ⁴ Laboratory of Socioecology and Social Evolution, Zoological Institute, University of
17 Leuven, Naamsestraat, 59, B-3000, Leuven, Belgium

18

19

20 *Author to whom correspondence should be addressed: Marco A. Del Lama

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22 **Running title:** Morphometric variation in *Partamona rustica* populations

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24

25 **Abstract**

26 Wing morphology has been used in taxonomic and systematic studies of insects, often
27 enabling the identification of groups based on variation. In this study, wing Geometric
28 Morphometrics was used to verify if eastern and western populations of *Partamona*
29 *rustica*, separated by the São Francisco River, are discriminated, thus confirming previous
30 molecular data. The two groups of *P. rustica* exhibited significant differences in wing
31 size and shape. Better differentiation of populations and groups was achieved with the
32 centroid size. We generated dendrograms using Mahalanobis and Procrustes distances,
33 which discriminated the eastern and western populations. Isolation by distance between
34 morphometric and geographic distances was found. The confirmation of the two
35 population groups points out the need of further studies investigating the occurrence of
36 barriers to gene flow and colonization history in the semiarid region by this stingless bee.

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39 **Keywords:** geometric morphometrics, population differentiation, stingless bees,
40 semiarid region, wing variation

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44 **Introduction**

45 The characterization of diversity and population structure is necessary to
46 implement properly conservation strategies (Frankham et al. 2010). The use of different
47 methods for estimating the degree of variation of traits and its distribution in populations
48 enables an accurate assessment of their genetic status, the influence of environmental
49 factors on the expression of phenotypic characteristics, and the adaptation of populations
50 to specific ecotypes (Hurtado-Burillo et al., 2016; Nunes et al., 2013; Francoy et al.,
51 2011). Such methods of diversity characterization also allow the identification of the
52 factors that may affect the size and shape of biological structures, such as geographic
53 barriers, climate, temperature, altitude, and genetic differences (Dellicour et al., 2017).

54 Pollinators are among the most important organisms for the maintenance of forest
55 ecosystems and stingless bees (Meliponini, Apidae) are one the most important bee
56 pollinators in the tropical and subtropical region (Grüter, 2020). In Brazil, 207 species of
57 stingless bees' genera have been described (Silveira et al. 2002), but the diversity in the
58 Neotropics reached to 426 species (Grüter, 2020). Assessing the diversity and population
59 structure of stingless bees using genetic and geometric morphometrics tools have been
60 successfully achieved (Francisco et al., 2008; Halcroft et al., 2016; Hurtado-Burillo et al.,
61 2016; Galaschi-Teixeira et al., 2018). Those studies allow the discrimination of
62 populations and are critical to understand the biodiversity of this group and point out
63 important areas of bees' conservation (Miranda et al., 2019). There is a need for more
64 comprehensive studies on the biology and conservation efforts directed at protecting
65 stingless bees' biodiversity in the Neotropics, as it is expected that stingless bee's
66 diversity is even richer than it has been described (Gruter 2020).

67 Stingless bees of the genus *Partamona* Schwarz 1939 are grouped into 32
68 recognized species (Camargo & Pedro, 2003, 2013; Pedro & Camargo, 2003) distributed

69 from southern Mexico to southern Brazil. *Partamona rustica* Schwarz 1939 is endemic
70 to the Cerrado (savanna) and Caatinga (xeric scrubland forest) biomes in Brazil. Those
71 biomes have suffered profound anthropogenic changes and the fauna and the flora require
72 further knowledge as a first step towards conservation (Klink & Machado, 2005; Zanella
73 & Martins, 2003). Although the geographic distribution of *P. rustica* was described to be
74 restricted from the northern portion of the state of Minas Gerais (Cerrado) to the
75 southwestern portion of the state of Bahia (Caatinga) (Camargo & Pedro, 2003), the
76 occurrence of colonies of this species 150 km north of this limit was reported (Miranda
77 et al., 2015). Interestingly, 90% of *P. rustica* nests sampled were associated with active
78 termite mounds built in the upper portion of trees, usually in association with
79 *Constrictotermes cyphergaster* Silvestri.

80 In a previous population genetics study of *P. rustica* with colonies from eleven
81 locations in the states of Bahia and Minas Gerais, it was demonstrated the existence of
82 two groups of genetically distinct populations based on sequences of four mitochondrial
83 genes and eight microsatellite loci (Miranda et al., 2016). The first group was composed
84 of two populations located western of the São Francisco River (SFR) and the second was
85 composed of nine populations located eastern of the river. Molecular analysis of variance
86 (AMOVA) based on gene sequences and microsatellite genotypes showed that much of
87 the variation is between groups (ϕ_{CT} = 0.468; ϕ_{CT} = 0.218, respectively), indicating high
88 and moderate differentiation between the two groups of populations. However, given the
89 difference in number of populations between the two groups, the authors stressed the need
90 for further studies as well as the use of a different analytical method to confirm the
91 findings.

92 The morphological variation of the wing has long been used in taxonomic and
93 systematic studies of insects, making it possible to identify species comparing only the

94 morphology of this structure (Pretorius, 2005). In recent years, studies on morphological
95 variation have predominantly employed geometric morphometrics (GM) of Cartesian
96 geometric coordinates rather than linear measurements (Tatsuta et al., 2018). A large
97 number of wasp and bee species has been studied in this way (Aytekin et al., 2007;
98 Tofilski, 2008; Owen, 2012; Falamarzi et al., 2016; Žikić et al., 2017; Dellicour et al.,
99 2017), and GM has been used as a taxonomic tool to discriminate from morphotypes and
100 lineages inside populations to genera of bees, particularly stingless bees (Francoy et al.,
101 2009; Nunes et al., 2013; Combey et al., 2013; Bonatti et al., 2014; Quezada-Euán et al.,
102 2015; Halcroft et al., 2016; Francoy et al., 2016; Galaschi-Teixeira et al., 2018; Santos et
103 al., 2019).

104 Therefore, the present study aimed to verify whether wing geometric
105 morphometrics discriminate *P. rustica* populations located at western and eastern sides
106 of SFR. In this work, we included more samples located at the west side of the SFR to
107 confirm the occurrence of the two groups. This would provide greater support to the
108 notion that the SFR may act as a barrier to gene flow among populations of *P. rustica*.
109 The establishment of possible proximal causes of interpopulation differentiation in this
110 bee species provides relevance to the present study.

111

112 **Material and methods**

113 *Sampling*

114 We obtained 161 colonies of *P. rustica* collected from 14 localities (Table S1 and
115 Figure S1). We used the 143 colonies from 11 localities previously analyzed (Miranda et
116 al., 2016) and we increased the sampling with 18 new colonies located at the western side
117 of the SFR (including the localities of Coribe, São Félix do Coribe, and Montalvânia).
118 Permits necessary for the fieldwork and collection of samples were issued by the Brazilian

119 Institute for Biodiversity Conservation (ICMBio) (number 31750). The field studies did
120 not involve endangered or protected species.

121 *Morphometric analysis*

122 Three adult workers from each of the 161 nests sampled at the 14 locations (12
123 located in the state of Bahia and two in Minas Gerais) were analyzed (Table S1, Figure
124 S1). The right forewing of each adult worker was removed and mounted between
125 microscope slides. Images were captured using a Leica S6D digital camera coupled to a
126 Leica DFC450 stereomicroscope. For the analysis of partial deformations, a database was
127 created using tps format to store the wing image information at the tpsUtil64 software,
128 version 1.79 (Rohlf, 2015).

129 To reduce possible human errors, seventeen landmarks (LM 9, 10, and 17 of type
130 III, *sensu* Bookstein 1991) were manually digitized twice (independently) at the wing
131 vein intersections using the tpsDig2 software, version 2.16 (Rohlf, 2015) (Figure S2). The
132 data used for analysis correspond to the average of the two markings performed on each
133 wing. In the MorphoJ software, version 1.6 (Klingenberg, 2011), the images underwent
134 Procrustes adjustment, enabling discriminant function analysis (DFA), principal
135 component analysis (PCA), and canonical variate analysis (CVA). ANOVA was
136 performed using two classifiers: i) considering the 14 populations independently and ii)
137 considering the two population groups (from the eastern and western sides of the SFR).
138 Kaiser-Meyer-Olkin (KMO) test was used in order to determine the suitability of the data
139 for the multivariate analyses. The function *KMO* in the package *psych* was used. The
140 overall Measure of Sampling Adequacy (MSA) was 0.5, meaning all coordinates may
141 reasonably be retained for the multivariate analyses.

142 Kruskal-Wallis one-way ANOVA on ranks was performed using the
143 STATISTICA 6.0 software (StatSoft 2001) to determine whether the mean values of

144 centroid size (CS) in the samples from the 14 populations were statistically different.
145 Kruskal-Wallis analyses among populations were assessed conducting pairwise
146 comparisons and Bonferroni correction for multiple comparisons. Additionally, a t-test
147 was performed to compare the mean centroid size in samples from eastern (occurring
148 predominantly in *Caatinga* areas) and western populations (predominantly in *Cerrado*
149 areas) of the SFR.

150 Mahalanobis and Procrustes distances were used to generate interpopulation
151 similarity dendrograms based on the neighbor-joining algorithm using the Mega software,
152 version 6.0 (Tamura et al., 2011). The correlation between morphological and geographic
153 distances, obtained using Google Earth version 6.1.0.5001 (Google Inc. 2011), was
154 verified using a Mantel test with the TFPGA software (Miller, 1997).

155 All software used in our morphometric analysis are freely accessible and available
156 at <http://life.bio.sunysb.edu/morph> and <http://www.flywings.org.uk/MorphoJ>.

157

158 **Results**

159 We analyzed 483 forewings of *P. rustica*. Based on the shape covariance data
160 matrix derived from the Procrustes fit, the PCA of the seventeen anatomical landmarks
161 revealed 30 components responsible for the total variation. The first 16 explained 92.16%
162 of the total variation, with the first two PC axis explaining 43.30% of the variation (PC1:
163 26.84%; PC2: 16.46%) (Figure S3). For data visualization, we showed the first two axes
164 of the CVA (Figure 1) explaining 66.1% of the total variance in the data (CV1: 42.2%;
165 CV2: 23.9%). The histogram of the first CV, obtained in the MorphoJ, showed a
166 separation of the two groups located western and eastern of the SFR, with some overlaps
167 (Figure S4).

168 The Mahalanobis distances between pairs of populations ranged from 2.1961
169 (TNO x MAC) to 7.2428 (IRA x MTV), whereas the Procrustes distances ranged from
170 0.0064 (MAC x ITU) to 0.0271 (CSI x SFC) (Table S2).

171 The discriminant function analysis resulted in 98.91%, demonstrating that nearly
172 all workers were correctly assigned to their original populations. Accuracy in the cross-
173 validation test was 87.16%.

174 Differences in size and shape of the wings of adult females of *P. rustica* were
175 observed. ANOVA performed considering the 14 populations independently generated a
176 statistically significant F ($P < 0.0001^{***}$) for centroid size (Figure 2) and shape (Table
177 1A). When populations were grouped into eastern and western groups, the ANOVA
178 results were equally significant ($P < 0.0001^{***}$) for centroid size and shape (Table 1B),
179 with a significant increase in the F value for wing shape as well as a marked decrease in
180 the F value for centroid size in this second approach.

181 The discriminant analysis correctly assigned 95.73% of the individuals to their
182 respective groups of origin (eastern or western), whereas the correct assignment rate in
183 the cross-validation was 94.17%. As the PCA analyzed the same covariance matrix of the
184 general data, the results for the two population groups were the same as described above
185 for the populations taken as independent units.

186 The Kruskal-Wallis one-way ANOVA on ranks showed that centroid values
187 differed significantly between populations ($H_{13} = 184.687$, $P < 0.001^{***}$). The
188 populations from Coribe and São Félix do Coribe (located western of the SFR) had higher
189 values of centroid size than the other populations. Comparing mean centroid size in the
190 two population groups, it appears that the females from the western group (predominantly
191 *Cerrado* area) have a significantly higher mean centroid value than females from the
192 colonies/populations from the east of the SFR (predominance of *Caatinga* areas) (Figure

193 S5), as confirmed by the Student's *t* test ($t = 8.512$, $df = 452$, $P < 0.001^{***}$; 95% CI =
194 0.121 to 0.0756).

195 The Mantel test revealed a positive correlation between geographic distances and
196 Mahalanobis ($r = 0.6117$; $P < 0.0001^{***}$) and Procrustes ($r = 0.5317$; $P < 0.0001^{***}$)
197 distances, which is consistent with the usually smaller Mahalanobis and Procrustes
198 distances between populations of the same group (eastern or western) and larger
199 Mahalanobis and Procrustes distances between populations of different groups (Table
200 S2), as the groups were separated by the São Francisco river which could impose a
201 geographical barrier. These results are reflected in the similarity dendrograms (Figure 3)
202 generated from the Mahalanobis and Procrustes distances, which show the separation of
203 populations into two groups, eastern and western populations of the SFR.

204

205 **Discussion**

206 Geometric morphometric analyses using wing size and shape discriminate the populations
207 of *P. rustica*. As we expected based on our previous population genetics study (Miranda
208 et al. 2016), the Mahalanobis and Procrustes distances discriminate eastern and western
209 populations of *Partamona rustica* separated by São Francisco River. The differentiation
210 of populations located on opposite banks of the San Francisco River was improved in the
211 present work with the inclusion of 18 colonies of *P. rustica* from three new sites located
212 to the western of the SFR, giving new support to the results previously reported based on
213 molecular data (Miranda et al., 2016).

214 Wing size is a good estimator of body size (Dellicour et al. 2017) and survival,
215 resource acquisition and reproductive success are generally size-dependent traits
216 (Takahashi & Blanckenhorn, 2015). Our analyses of wing size assessed by centroid size
217 significantly exhibited higher variation among populations of *P. rustica* than wing shape

218 (Table 1A). This finding was expected, as size variation in insects is related to the quality
219 and quantity of food given to the larvae (Peruquetti, 2003; Campos et al., 2018), and to
220 the foraging range of colonies (Veiga et al., 2013). Colony fitness in stingless bees is
221 highly associated with the variation in worker body size, which is dependent on trophic
222 resources stored within the colony, as at least 75% of the variation are attributed not to
223 phylogenetic effects but rather to food requirements (Pignata & Diniz-Filho, 1996). Thus,
224 as wing size variation may reflect the availability of trophic resources in each
225 environment, this makes sense as the supply dynamics certainly vary temporally in the
226 *Cerrado* and *Caatinga* biomes (Campos et al., 2018; Miranda et al., 2021), which have
227 distinct characteristics in terms of terrain, climate and vegetation composition, as well as
228 the idiosyncratic evolutionary history of *P. rustica* (see Miranda et al., 2015; Miranda et
229 al., 2016). Alternatively, this variation in body size has been also associated with a
230 different response of these populations to specific features of their local environments
231 (Grassi-Sella et al., 2018; Ribeiro et al., 2019).

232 Canonical variate analyses revealed little overlap between samples of the two
233 groups. A broader range of variation was found among individuals from the eastern group
234 compared to those from the western group is consistent with the larger number of colonies
235 from the eastern side as well as the larger number of haplotypes identified ($n = 22$)
236 compared to the western group ($n = 8$) (Miranda et al., 2016). However, these authors
237 found a different result regarding the microsatellite data, showing greater genetic
238 diversity of these markers among populations in the western group despite the small
239 number of populations on this side of the SFR (Miranda et al., 2016). The agreement
240 between the similarity dendrograms and the characterization of the two groups previously
241 defined by population genetic analysis denotes a high resolution of geometric

242 morphometrics to capture subtle differences in wing size and shape between close groups,
243 corroborating with other studies in bees (Quezada-Euán et al., 2015; Francoy et al., 2016).

244 In the analysis of variance (ANOVA) performed with Procrustes distances the
245 estimated F was greater for centroid size than shape of the wing of *P. rustica* workers.
246 The large reduction in the F value for centroid size and the large increase in the F value
247 for shape when the analysis was performed with the two groups compared to the analysis
248 considering the 14 populations independently is noteworthy. These results seem to be
249 coherent, since a large variation in centroid size is expected in samples from different
250 locations (first analysis considering the 14 populations) due to the different environments
251 to which the respective local colonies are exposed. When analyzing the two groups
252 (second analysis), the F value decreases because we are now comparing the average of
253 the environments experienced by the populations east and west of the SFR. On the other
254 hand, the significant increase in the F value found in the two groups can be attributed to
255 the differences in genetic composition between population groups demonstrated
256 previously (Miranda et al., 2016) and analyzing the shape of the wing in the present study.
257 Ontogenetic aspects linked to maternal and feeding effects (Peruquetti, 2003), climate
258 and temperature may be directly related to size variation among individuals from different
259 populations and may be inducing adaptive responses in these populations (Nunes et al.,
260 2013). While environmental factors certainly contribute to the differences in size, the
261 shape of a structure is a trait of greater complexity that involves environmental and
262 genetic factors that are subject to developmental constraints (Klingenberg, 2016). So,
263 shape differences may be linked to local adaptive responses to foraging behaviors, flight
264 dynamics and different environments to which populations are exposed (Benítez et al.,
265 2013; Nunes et al., 2013).

266 The Mantel test supports the association between the variation in wing
267 morphology and biotic as well as abiotic factors by revealing a positive correlation
268 between morphological and geographic distances. However, one cannot rule out possible
269 effects of phenotypic plasticity on the expression of individual characteristics (Nunes et
270 al., 2013). Isolation by distance – reported here for morphometric data and elsewhere for
271 microsatellite data (Miranda et al., 2016) – also explains some reduction in gene flow.
272 Other factors, such as the mode of reproduction in Meliponini colonies, with the
273 temporary dependence of daughter colonies on the mother colony (Nogueira-Neto, 1954;
274 Engels & Imperatriz-Fonseca, 1990; Cronin et al., 2013), a small flight radius (Wille &
275 Orozco, 1975; Araújo et al., 2004; but see Zayed et al., 2005 about the distance wild bees
276 cover during foraging trips is related to their dispersal ability) and geographic barriers (in
277 this case, the SFR valley and/or different phytophysiognomies) are additional factors that
278 can lead to evolutionary processes that differentiate populations over time (May-Itzá et
279 al., 2012; Hurtado-Burillo et al., 2016). As males, to some extent, ensure the dispersion
280 of genes in populations, a certain level of genetic homogeneity must be produced, which
281 is consistent with the moderate gene flow estimated by the microsatellite loci (Miranda
282 et al., 2016) and the low overlap found between population groups in the geometric
283 morphometric analysis.

284 According to our previous work (Miranda et al., 2016), the eastern and western
285 groups split recently – at the end of the Pleistocene about 102 kya (95% HPD: 45,588–
286 979,902 kya) – and phylogenetic reconstruction indicated that *P. rustica* possibly
287 originated western of the SFR valley, with the subsequent colonization of the eastern
288 region. The SFR or isolation by distance may be affecting gene flow and isolating the
289 populations studied here. This argument is supported by the fact that this region has low
290 potential for the historical occurrence of the species, as seen in the ecological niche

291 modeling analysis, as well as for the termite that builds the substrate in which this bee
292 nest (Miranda et al., 2015). On the other hand, in a study using geometric morphometrics
293 on populations of *Melipona mandaçaia*, which is a stingless bee that also occurs in
294 *Caatinga* areas along the São Francisco River, it was found no evidence that the valley
295 or the river itself was a barrier to gene flow between populations sampled on the different
296 banks of the river (Prado-Silva et al., 2016). Alternatively, the differences between the
297 western (predominantly *Cerrado*) and eastern (predominantly *Caatinga*)
298 phytophysiognomies of the SFR may be valid arguments for the differentiation between
299 the groups observed herein, as differences in rainfall, floral types, substrates and altitudes
300 between the two regions may be limiting factors for the occurrence of the species, which
301 has preferences for certain environments and substrates (termite nests) (Miranda et al.,
302 2015).

303 The geometric morphometric differences between the two groups of populations
304 provide support to the hypothesis that *P. rustica* originated in areas of the *Cerrado* and
305 subsequently colonized areas of the *Caatinga* (Miranda et al., 2016). Thus, the previous
306 hypothesis that the genus *Partamona* originated in Amazon and Andean areas (Camargo
307 & Pedro, 2003), along with evidence that the biota of the *Cerrado* is more related to the
308 biota found in the Amazon (Werneck et al. 2011) support the idea that the group located
309 western of the SFR is older and gave rise to the eastern group.

310 Concluding, geometric morphometrics was effective in demonstrating differences
311 in wing size and shape among colonies of *P. rustica* from different geographical locations.
312 The geometric morphometric data further supported the distinction between the two
313 groups of *P. rustica* previously suggested by genetic analysis based on molecular markers
314 (mtDNA and microsatellite loci). Despite the new evidence, it is not possible to confirm
315 that the São Francisco River constituted an effective geographic barrier that induced

316 genetic differentiation processes, especially regarding wing shape, as the level of genetic
317 isolation among populations in the two groups was not estimated, considering the possible
318 recent separation between them. However, the observed geometric morphometric
319 variation appears to be, at least partially, the result of interpopulation genetic variation.
320 Thus, subsequent studies are needed to assess how much of this variation may be
321 attributed to genetic and/or environmental factors as well as determine the possible
322 meaning of this variation, which is an under explored subject in bees, despite being one
323 of the central goals of evolutionary biology.

324

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338 **ORCID**

339 Tiago Maurício Francoy: 0000-0002-2413-966X

340 Elder Assis Miranda: 0000-0003-4679-3686

341 Cíntia Akemi Oi: 0000-0002-3682-8219

342 Kátia Maria Ferreira: 0000-0003-1387-7066

343 Marco Antonio Del Lama: 0000-0002-3329-8953

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562 **Tables**

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564 **Table 1.** (A) ANOVA table including Procrustes distances for centroid size (CS) and
 565 shape (SH) using *Partamona rustica* wings considering the 14 populations. (B)
 566 ANOVA table including Procrustes distances for centroid size (CS) and shape (SH)
 567 using *P. rustica* wings considering the two groups (western and eastern side of SFR).
 568 Sum of squares (SS); mean squares (MS); degrees of freedom (dF). The F values have P
 569 < 0.0001***.

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A - ANOVA (considering the 14 populations)					
	Effect	SS	MS	dF	F
CS	Individual	28951189.154814	2227014.550370	13	217.7
	Residual	4778672.443366	10254.661896	466	
SH	Individual	0.06004763	0.0001539683	390	13.35
	Residual	0.16124608	0.0000115341	13980	

B - ANOVA (considering Western x Eastern populations)					
	Effect	SS	MS	dF	F
CS	Individual	6199655.653226	6199655.65322	1	107.6
	Residual	27530205.944955	57594.573107	478	
SH	Individual	0.02122182	0.0007073941	30	50.7
	Residual	0.20007189	0.0000139520	14340	

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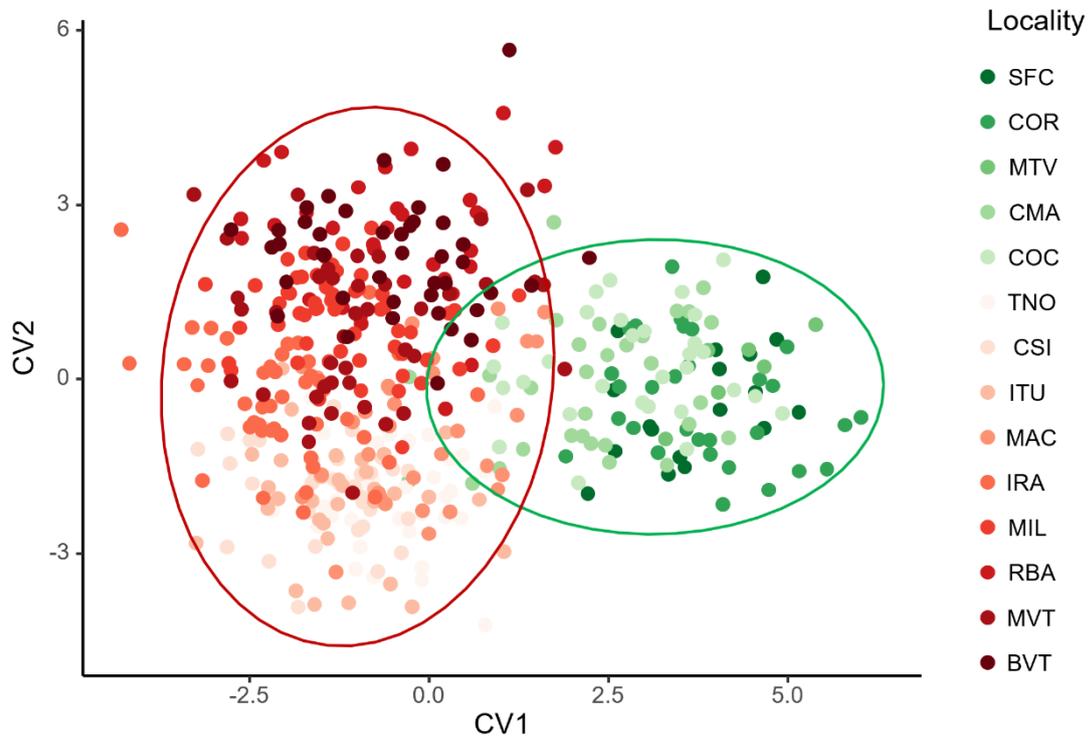
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582 **Figures**

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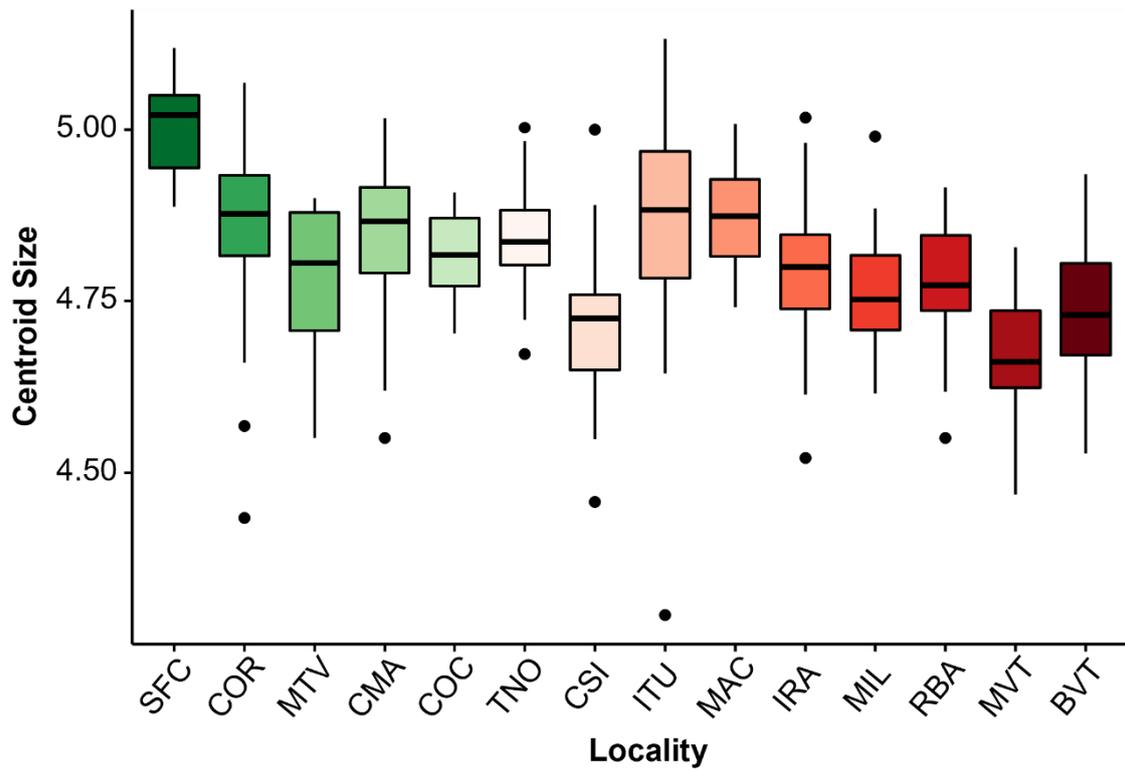
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586 **Figure 1.** Scatter plot of the fourteen populations of *Partamona rustica* analyzed,
587 generated from the first two canonical variate analyses (CVA). Ellipses indicated 95%
588 confidence intervals. The green circles represent the western populations and the red
589 circles the eastern populations.

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594 **Figure 2.** Boxplot of the wing centroid sizes for each population of *Partamona rustica*.

595 Vertical bars represent 95% confidence intervals. Pairwise comparisons among

596 populations are indicated by the letters of significance: SFC (a); COR (bcd); MTV

597 (bcdefg); CMA (bcd); COC (bcd); TNO (bcd); CSI (ef); ITU (bc); MAC (b); IRA (cdg);

598 MIL (eg); RBA (deg); MVT (f); BVT (efg). The green colors represent the western

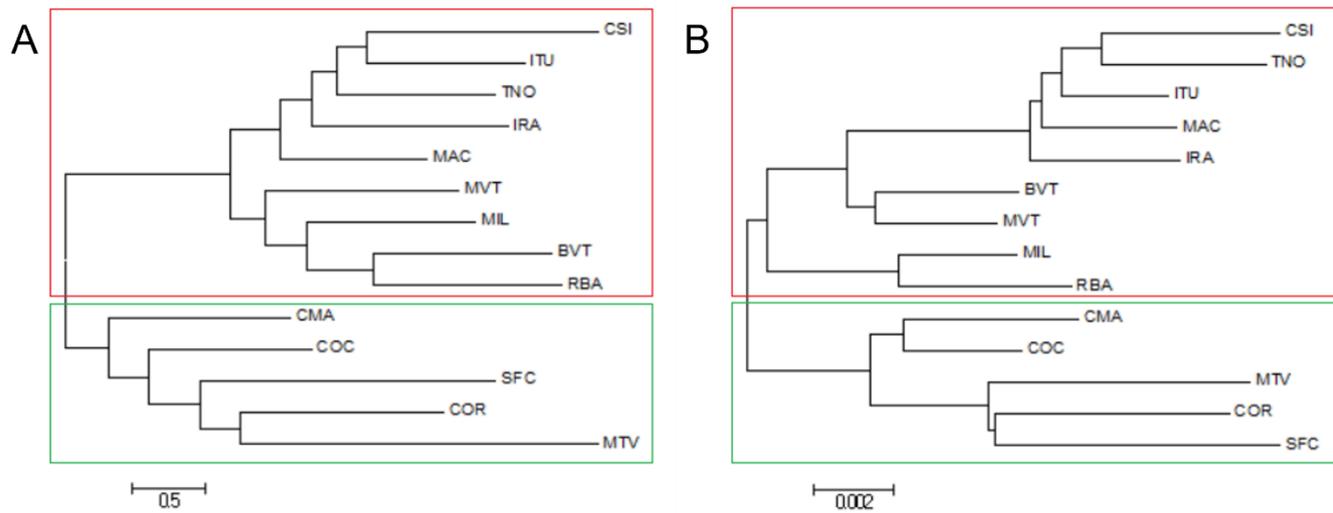
599 populations and the red colors the eastern populations. (median \pm quartiles; whiskers: data

600 range; circles: outliers).

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605 **Figure 3.** Neighbour-joining dendrogram showing the relationships of similarity among
606 populations of *Partamona rustica* based in Mahalanobis (A) and Procrustes (B) distances
607 (in green, western group and in red eastern group).

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