

1 **Adaptation of *Drosophila subobscura* chromosomal inversions to**
2 **climatic variables: The Balkan natural population of Avala**

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22 **Abstract.**

23 The adaptive value of chromosomal inversions continues raising relevant questions in
24 evolutionary biology. In many species of the *Drosophila* genus, different inversions
25 have been recognized to be related to thermal adaptation, but it is necessary to
26 determine to which specific climatic variables the inversions are adaptive. With this
27 aim, the behavior of thermal adapted inversions of *Drosophila subobscura* regarding
28 climatic variables was studied in the natural population of Avala (Serbia) during the
29 2014-2017 period. The results obtained were compared with those previously reported
30 in the Font Gropa (Barcelona, Spain) population, which presents different climatic and
31 environmental conditions. In both populations, it was observed that most thermal
32 adapted inversions were significantly associated with the first, second or both principal
33 components, which were related with maximum, minimum and mean temperatures.
34 Moreover, a significant increase over years (2004-2017) for the minimum temperature
35 was detected. In parallel, a significant variation over time in Avala was only observed
36 for the frequencies of ‘warm’ and ‘non-thermal’ adapted inversions of the U
37 chromosome. However, stability in the chromosomal inversion polymorphism was
38 observed for the 2014-2017 period which might result from the temporal span of the
39 study and / or selective process acting on the population.

40

41 **Key words** *Drosophila* · chromosomal inversions · adaptation · temperature · selection

42 **Introduction**

43 Chromosomal inversions were detected one century ago by Sturtevant (1921),
44 and Dobzhansky observed that their genetic content presented adaptive properties (for a
45 revision see Lewontin et al. 1981). The presence of inversions is a karyotypic
46 characteristic of a large number of species across the animal kingdom, and this
47 structural chromosomal change is a key element in evolutionary processes such as
48 adaptation to diverse environmental conditions or speciation (Sperlich and Pfriem 1986;
49 Hoffmann et al. 2004; Feuk et al. 2005; Ayala and Coluzzi 2005; Hoffmann and
50 Rieseberg 2008; McAllister et al. 2008; Kirkpatrick 2010; Nie et al. 2012; Fuller et al.
51 2018; Kapun and Flatt 2019). One of the best studied cases is the adaptive potential of
52 chromosomal inversion polymorphism of *Drosophila subobscura*. For European
53 researchers, this species is somehow the equivalent of *D. pseudoobscura* for North
54 American evolutionary scientists. Nevertheless, *D. subobscura* has the advantage that
55 presents inversions in all chromosomes of its karyotype with the exception of the dot
56 (for a revision see Kunze-Mühl and Sperlich 1955; Krimbas 1992, 1993). Interestingly
57 in the Palearctic populations of this species, it was detected that the frequencies of
58 inversions (or their combinations, the so-called arrangements) presented clines
59 correlated with latitude (Krimbas and Loukas 1980; Krimbas 1992, 1993). Although
60 different explanations were possible, the discovery of latitudinal cline variation of
61 chromosomal inversion frequencies in the North and South American colonizing
62 populations of *D. subobscura* which exhibit the same sign than that found in the Old
63 World, strongly supported the adaptive role of inversions (Prevosti et al. 1988, 1989;
64 Ayala et al. 1989). To elucidate the adaptive meaning of *D. subobscura* inversions in
65 Palearctic populations, Menozzi and Krimbas (1992) analyzed their possible
66 relationship with many environmental variables. It must be remembered that *D.*

67 *subobscura*, as a species, can only survive and reproduce under certain climatic
68 conditions. This fact was clearly demonstrated by noting the distribution of *D.*
69 *subobscura* in the Palearctic region and in those colonized areas of America (North and
70 South). In these three zones, a parallel and gradual variation of climatic conditions
71 (western maritime, Mediterranean and semiarid/arid) can be observed (The Times 1972;
72 Prevosti et al. 1988, 1989). Even in a particular location and in the same season, the
73 proportion over years of *D. subobscura* with regard to other species of the *Drosophila*
74 genus depends on the weather conditions (Argemí et al. 1999, 2003; Galludo et al.
75 2020).

76 Changes in the inversion chromosomal polymorphism over time in natural
77 populations of this species regarding global warming have been extensively studied
78 (Orengo and Prevosti 1996; Rodriguez-Trelles and Rodriguez 1998; Solé et al. 2002;
79 Balanyà et al. 2004, 2006, 2009; Rego et al. 2010; Dolgova et al. 2010; Zivanovic et al.
80 2012, 2015; Orengo et al. 2016). From early studies, inversions were classified as
81 ‘cold’, ‘warm’ or ‘non-thermal’ adapted (Menozzi and Krimbas 1992; Rodriguez-
82 Trelles et al. 1996; Rego et al. 2010; Zivanovic et al. 2016). However, the adaptive
83 response of particular inversions in face of different climatic variables deserves to be
84 better analyzed. Recently, a detailed study was carried out in the Font Grogà
85 (Barcelona) populations, where the relation of different inversions with regard to
86 climatic variables (mean, maximum and minimum temperatures, humidity and rainfall)
87 was analyzed (Galludo et al. 2018). The most relevant result of that research was that
88 ‘cold’ inversions (A_{st} , J_{st} and E_{st}) increased in frequency when T_{max} (maximum
89 temperature) and T_{min} (minimum temperature) decreased, whereas the ‘warm’
90 inversions (E_{1+2+9} , O_{3+4+1} and O_{3+4+8}), augmented in frequency with an increase in T_{max}
91 and T_{min} . Furthermore, inversions seem to adapt not only to temperature, but also to

92 humidity (Hm) and rainfall (Rf). For these reasons, it would be evolutionary relevant to
93 compare the behavior of thermal adapted inversions in another different population of
94 *D. subobscura* with regard to climatic conditions, season, different habitat and historic
95 factors (another refugium of last glaciations).

96 The present research is articulated in two objectives regarding the thermal
97 adapted chromosomal inversions of *D. subobscura*. The first objective is the main and
98 aims to deepen the knowledge of the relationship between particular chromosomal
99 inversions and some climatic variables. For this purpose, the chromosomal
100 polymorphism of the Balkan *D. subobscura* population of Mount Avala (Serbia) was
101 analyzed during four consecutive years. This population was previously studied
102 (Zivanovic and Mestres 2010; Zivanovic et al. 2014a, 2015, 2016) and could be very
103 useful because it shows a different chromosomal polymorphism composition of that
104 from Font Gropa (Galludo et al. 2018; Zivanovic et al. 2016). Furthermore, both
105 populations seem to differ regarding the climate and vegetation. Finally, the Iberian
106 Peninsula and the Balkans are considered biodiversity refugia during the Pleistocene
107 glaciations adding historical processes in their differentiation (Taberlet et al. 1998;
108 Hewitt 2000; Tzedakis 2004; Provan and Bennett 2008; Wielstra and Arntzen 2020).
109 Thus, Avala and Font Gropa *D. subobscura* populations would be differentiated due to
110 different historic and adaptive process. For these reasons, to compare both populations
111 regarding the effects of climatic variables on the inversion frequencies could improve
112 the knowledge of the role of chromosomal inversions on adaptation. To achieve this
113 aim, our research was organized as follows: first, the possible differences in
114 chromosomal inversions composition and abundance between Avala and Font Gropa
115 were analyzed. Also, a comparison between both populations with regard to some
116 climatic variables was carried out. Third, a qualitative contrast between the vegetation

117 of these two localities was made. Once detected the possible differentiation between
118 both populations, a complete analysis to elucidate the effect of some climatic variables
119 on different inversions in Avala population was computed. The results were compared
120 with those from Font Gropa. This comparison should allow to obtain more information
121 on a relevant question: will the chromosomal inversions (those that are the same and
122 those that are not) in different biological and physical conditions react in a similar
123 adaptive way with respect to variations in some climatic variables?

124 The other objective is secondary, but related to first one, taking the advantage
125 that Avala population was previously studied several years ago (Zivanovic and Mestres
126 2010; Zivanovic et al. 2015). It seeks to answer the following question: will thermally
127 adapted inversions increase in frequency as our planet's global warming augments, or is
128 there a maximum threshold for these frequencies? For this purpose, the chromosomal
129 inversion data from the last year of the present research (2017) was compared with
130 those available from Avala recorded in 2004 and 2011 (Zivanovic and Mestres 2010;
131 Zivanovic et al. 2015) to analyze the changes over time in 'cold', 'warm' or 'non-
132 thermal' adapted inversion frequencies. In parallel, the behavior of some climatic
133 variables in Avala for the period 2004-2017 was analyzed to explore the potential global
134 warming effect.

135

136 **Materials and Methods**

137 ***D. subobscura* collections and preparation of chromosomes**

138 Flies were collected from Mount Avala population (Serbia, 44°41'25"N
139 20°30'51"E), located at 18 km south of Belgrade. They were sampled from a forest with
140 polydominant communities of *Fagetum submontanum mixtum* that is approximately

141 450 m a.s.l. Collections were obtained strictly in the same place in 2014 (from the 10th
142 to the 22nd of June), 2015 (from the 1th to the 14th of July), 2016 (from the 10th to the
143 18th of June) and 2017 (from the 9th to the 19th of June). In all samples, flies were netted
144 from 4 p.m. to 8 p.m. using 40 fermenting apple baits.

145 Wild males and sons of wild females were crossed in individual vials with virgin
146 females from the Kussnacht reference strain. Sample sizes are presented in Table 1.
147 This was homokaryotypic for the standard arrangements in all five chromosomes (A, E,
148 J, U and O). To obtain the chromosomal preparations third instar larvae from the F₁
149 were dissected and polytene chromosomes were stained and squashed in aceto-orcein
150 solution. To properly identify the inversions, the chromosomal maps of Kunze-Mühl
151 and Müller (1958) and Krimbas (1992, 1993) were used. To achieve the karyotypes
152 with a probability higher than 0.99, at least eight larvae were analyzed from the progeny
153 of each cross. All crosses were carried out at 18°C, 60% relative humidity and 12h/12h
154 light/dark cycle. Finally, chromosomal inversions were classified as ‘C’ (cold adapted),
155 ‘W’ (warm adapted) and ‘N’ (non-thermal adapted) following the criteria of Menozzi
156 and Krimbas (1992), Rego et al. (2010) and Zivanovic et al. (2016).

157 For the aim of comparison, the Font Gropa (Barcelona, 41°25'54"N 2°07'20"E)
158 population is situated on the foothills of the Tibidabo mountain (on the edge of
159 Barcelona city at 415 m a.s.l.). Flies were collected in a forest of pines (*Pinus pinea*)
160 and ilexes (*Quercus ilex*) with Mediterranean brushwood, during the autumn of five
161 consecutive years (2011-2015) (Galludo et al. 2018).

162

163 **Climatic data and statistical analyses**

164 The Serbian Republic Hydrometeorological Service provided the meteorological
165 data from Avala (mean, maximum and minimum temperatures; dawn, noon, dusk and

166 mean humidity; rainfall). Temperature, humidity and rainfall were measured in
167 centigrade degrees, percentage and millimeters of precipitation, respectively.

168 All statistical computations were carried out using the *basic* and *vegan* packages
169 of R language (R Development Core Team 2014). To compare the chromosomal
170 composition between different years and departure of the observed frequencies of
171 chromosomal karyotypes from expectations, Fisher's exact test was used (statistically
172 significant $P < 0.05$). The corresponding P values were obtained using a bootstrap
173 procedure (100,000 runs). In all cases of multiple comparisons, the FDR correction
174 (Benjamini and Hochberg 1995) was applied, and it was reported as significant for $P <$
175 0.05 . The index of free recombination (*IFR*) was computed according to Carson (1955).
176 Using all *D. subobscura* chromosomes (A, E, J, U and O), a comparison between the
177 annual samples from Avala (2014-2017) and Font Gropa (2011-2015) populations was
178 carried out as follows. With this set of populations using the Bhattacharyya distance
179 (Bhattacharyya 1946), a Principal Coordinate Analysis was performed according to
180 Balanyà et al. (2006) and Mestres et al. (2009). Moreover, a cluster was computed with
181 the same data using the GEVA-Ward procedure, because it is considered excellent for
182 chromosomal inversion data (Irigoiien et al. 2010; Zivanovic et al. 2016). To measure
183 how faithfully the cluster preserved the pairwise distances between the original data, the
184 Pearson cophenetic correlation was computed.

185 To compare the climatic variables (mean, maximum and minimum temperatures;
186 dawn, noon, dusk and mean humidity; rainfall) between Avala and Font Gropa during
187 the same period of years (2014-2017) and using the values from May (the month before
188 trapping the flies) a Mann-Whitney U test was computed. Furthermore, a Ward cluster
189 was computed.

190 The relationship between chromosomal inversions and climatic variables (mean,
191 maximum and minimum temperatures, mean humidity and rainfall) was computed only
192 for those inversions observed in all Avala samples. For this reason, the infrequent
193 inversions J_{3+4} , U_1 , U_{1+2+3} , O_7 , O_{22} , O_{3+4+2} , O_{3+4+7} and O_{3+4+17} were excluded of the
194 analysis. Moreover, we gathered together the meteorological information for March,
195 April and May during the studied period (2014-2017), because we assumed that the
196 climate of these previous months before collecting the *D. subobscura* individuals could
197 considerably influence the chromosomal composition of the samples. The mean of the
198 values obtained for the climatic variables during these three months was used in the
199 calculations (Supplementary Table S1). Using the climatic variables, a Principal
200 Component Analysis (PCA) was carried out. In this way, the principal components
201 synthesize the information provided by the climatic variables. For each chromosome
202 and inversion, we considered a Poisson regression model taking as regressors these
203 principal components. For the individual significance of each component, the FDR
204 correction (Benjamini and Hochberg 1995) was applied and it was reported as
205 significant for $P < 0.05$. Moreover, we assessed the ratio of variation in abundance for
206 the chromosomal inversions when all climatic variables were kept constant and only
207 one variable was either increased or decreased in only one unit.

208 Finally, in the Avala population the possible global warming effect was
209 measured using data for mean, maximum and minimum temperatures, humidity and
210 rainfall recorded in the period 2004-2017 for the average of months of March, April and
211 May, because they were the months before the *D. subobscura* collections. For each
212 climatic variable, a temporal series was computed. Differences between frequencies of
213 ‘cold’, ‘warm’ and ‘non-thermal’ adapted inversions for the samples of 2004, 2011 and
214 the period 2014-2017, were studied using the Fisher exact test, as previously explained.

215 The thermal adaptation of the whole karyotype was measured using the *CTI* index
216 (Arenas et al. 2018).

217

218 **Results**

219 **Climate and vegetation comparisons between Avala and Font Gropa**

220 Regarding the kind of climate, both populations differ: Avala is classified as
221 western maritime, whereas Font Gropa is Mediterranean (The Times 1972; Prevosti et
222 al. 1988). This translates in different kind of natural landscapes: Avala belonging to a
223 forests and grasslands of temperate continental regions and Font Gropa to a typical
224 Mediterranean one. These features and historic factors during the Pleistocene
225 conditioned the vegetation of both localities. In the case of Avala, it is composed of
226 submontanum beech wood where *Fagus moesiaca* is the dominant tree together with
227 three other linden species (*Tilia argentea*, *Tilia cordata*, *Tilia platyphyllos*) and
228 brushwood plants (*Rubus hirtus*, *Pulmonaria officinalis*, *Asperula odorata*, *Carex*
229 *silvatica*, *Lamium galeobdolon* and others). In the Font Gropa site, the vegetation is
230 mainly composed of pines (*Pinus pinea*) and ilexes (*Quercus ilex*) with brushwood
231 (*Arbutus unedo*, *Ruscus aculeatus*, *Erica arborea*, *Hedera helix*, *Rubus ulmifolius*,
232 *Smilax aspera*, *Laurus nobilis* and others). Using the U Mann-Whitney test and
233 applying the FDR correction, differences were not detected in the climatic variables
234 when compared Avala and Font Gropa populations for the period 2014-2017 (T_{max} , P
235 = 0.6650, adjusted P = 0.8817; T_{min} , P = 0.7715, adjusted P = 0.8817; T_{mean} , P = 1.0,
236 adjusted P = 1.0; Rainfall, P = 0.0304, adjusted P = 0.1215; Dawn Hum, P = 0.0294,
237 adjusted P = 0.1215; Noon Hum, P = 0.0591, adjusted P = 0.1575; Dusk Hum, P =
238 0.1939, adjusted P = 0.3879; Mean Hum, P = 0.4705, adjusted P = 0.7528). However,

239 considering the *P* values without FDR correction a certain trend of differentiation was
240 observed for rainfall and two measures of humidity (dawn and noon). Finally, the
241 cluster analysis generated three groups: one containing only the Avala sample of 2014,
242 another with the remaining collections from Avala, and the last group with all Font
243 Groga samples (Supplementary Fig. S1). The cophenetic correlation coefficient was
244 0.954, indicating that the tree is a trustworthy reproduction from the distances used. For
245 the studied climatic variables, the cluster result seems to support a certain differentiation
246 between these two localities.

247

248 **The chromosomal inversion polymorphism of Avala population**

249 Focusing in the chromosomal polymorphism, the frequencies for each inversion
250 for the years 2014-2017 in Avala population are presented in Table 1. It is worth noting
251 that the J_{3+4} inversion, generally associated with arid climates (Krimbas 1992, 1993),
252 was detected in Avala for the first time in 2015. Likely, infrequent inversions U_1 , O_7
253 and O_{22} are a product of very rare recombination events. The U_{1+2+3} inversion was not
254 reported in Avala until 2017 and its frequency was negligible. It is usually found in
255 Southern Italy, Israel and Iran (Krimbas 1992). The O_{3+4+2} and O_{3+4+17} were observed for
256 the first time in Avala in 2011, both at very low frequencies. The first one is found at
257 low frequencies in different populations of the Palearctic region (Krimbas 1992;
258 Galludo et al. 2018; Madrenas et al. 2020), but in high frequencies in American
259 colonizing populations (Prevosti et al. 1988; Balanyà et al. 2003). The second is
260 distributed in the Iberian Peninsula at very low frequency (Solé et al. 2002; Mestres et
261 al. 1998; Galludo et al. 2018). The distribution of karyotypes in the samples of Avala
262 population is presented in Table 2. There were no deviations from the Hardy-Weinberg
263 equilibrium for any chromosome in any of the years analyzed (Supplementary Table

264 S2). Regarding the *IFR* values, they are very similar in the 2014-2017 samples,
265 indicating an equivalent amount of chromosomal polymorphism. The values estimated
266 correspond to those of populations located in the central area of *D. subobscura*
267 population (Krimbas 1992).

268

269 **Comparison between the chromosomal polymorphisms of Avala and Font Gropa**

270 Qualitative differences can be observed between the chromosomal
271 polymorphisms of Avala and Font Gropa regarding the type and abundance of
272 inversions. The multivariate analyses (Principal Coordinate Analysis and cluster)
273 demonstrated the existence of a differentiation (Fig. 1). In the PCoA, first, second and
274 third axes explained 67.75%, 20.52% and 5.44%, respectively. The samples from both
275 populations are clearly separated in the graphical representation (Fig. 1a). A similar
276 result can be observed in the cluster analysis (Fig. 1b), where two groups are defined,
277 one for the Avala samples and the other for those from the Font Gropa. The cophenetic
278 correlation coefficient was 0.902, which is interpreted as that the tree accurately
279 describes the genetic distances between the samples used.

280

281 **Relations between inversions and climatic variables**

282 The first three principal components (PC1, PC2 and PC3) obtained using the
283 climatic variables account for 57.52%, 38.49% and 3.99% of the variance, respectively.
284 Thus, using the three components we conserve the 100% of the explained variability
285 and no information is lost. PC1 is mainly related with temperature (*T*_{mean}, *T*_{max} and
286 *T*_{min}) and a certain effect of *R*_f is also observed; PC2 is dependent on *H*_m, *R*_f and
287 *T*_{min}; and finally, the effects of all variables are almost negligible for PC3 (Table 3).
288 The individual significance of PC1, PC2 and PC3 for the inversions is presented in

289 Table 4. The A_{st} , J_1 , U_{1+2} , E_{1+2+9} , O_{3+4} inversions were significant for both, PC1 and
290 PC2, being all classified as ‘warm’ with the exception of A_{st} (‘cold’). On the other
291 hand, the A_2 , E_{st} and $E_{1+2+9+12}$ inversions, considered respectively as ‘warm’, ‘cold’ and
292 ‘warm’, were significant for both, PC1 and PC3. Significance only for PC1 was
293 detected for the O_{3+4+22} inversion, being considered as ‘non-thermal’. The U_{1+2+6} , U_{1+8+2}
294 and O_{st} inversions were significant only for PC2, being considered as ‘non-thermal’,
295 ‘warm’ and ‘cold’, respectively. Thus, at least one principal component is significant for
296 most thermal adapted inversions with the only exception of A_1 , J_{st} , U_{st} , O_{3+4+1} and O_{3+4+8}
297 inversions. When all variables were kept constant, but only one variable of interest
298 changed by 1 unit, the ratios of variation in quantity of inversions for each chromosome
299 is presented in Table 5. In general, the studied environmental variables produced small
300 changes in the abundance of inversions. The $E_{1+2+9+12}$ inversion (‘warm’) presented and
301 increase with temperatures and a decrease with rainfall, whereas the O_{3+4+6} (‘non-
302 thermal’) presented only an increase with temperatures.

303

304 **Changes over time of the Acala chromosomal polymorphism**

305 The climatic variables studied during 2004-2017 presented annual variations
306 (Supplementary Fig. 2). Although all temperatures increased over time, only T_{min} was
307 significant ($P = 0.037$, with a slope of 0.092). Large fluctuations were observed for
308 humidity with a trend to decrease over time, although it was not significant. Finally, the
309 rainfall showed the expected erratic distribution.

310 All comparisons between different years and chromosomes (A, J, U, E and O),
311 but considering ‘cold’, ‘warm’ and ‘non-thermal’ adapted inversions are presented in
312 Supplementary Table S3. It is worth to remember that there are not ‘non-thermal’
313 adapted inversions for the A chromosome. Significant values were observed only for the

314 'warm' and 'non-thermal' adapted inversions of the U chromosome when comparing
315 the years 2004 with 2011 and 2004 with 2017. The variations of 'cold', 'warm' and
316 'non-thermal' adapted inversions over time are shown in Figure 2. The 'cold' inversions
317 presented a certain pattern of variation in different directions depending on the
318 chromosome during the interval 2014-2016, to return to levels similar to those of 2004
319 (Fig. 2a). Regarding the 'warm' inversions, their behavior is such as that of the 'cold'
320 inversions, with the clear exception of those from the U chromosome, which frequency
321 was increasing from 2004 to 2011 and then seemed to suffer only small fluctuations
322 (Fig. 2b). Finally, the changes of the 'non-thermal' inversion over time were
323 insignificant, with the exception of those from the U chromosome showing the opposite
324 behavior of the 'warm' inversions for the same chromosome (Fig. 2c). In summary, it
325 seemed that the increase of U chromosome 'warm' inversions would be at the expense
326 of the 'non-thermal' for the same chromosome.

327 The *CTI* values for Avala in 2014, 2015, 2016 and 2017 were 0.383, 0.262,
328 0.302 and 0.371, respectively. In general, these values are rather similar, but a little bit
329 lower, than those reported in the same population in 2004 (0.374) and 2011 (0.426)
330 (Arenas et al. 2018). All possible statistical comparisons between these *CTI* values were
331 computed and no evidence of significance was observed (Supplementary Table S4).

332

333 **Discussion**

334 There is still a gap between the information provided by the molecular studies of
335 inversions and their phenotypes regarding the adaptation to environmental conditions.
336 The *in situ* hybridization and genomics studies have been able to identify many
337 structural genes located inside chromosomal inversions of the *Drosophila* genus (for
338 instance, Clark et al. 2007; Laayouni et al. 2007; Guillén and Ruiz 2012; Fabian et al.

339 2012; Orengo et al. 2017; Reis et al. 2018; Karageorgiou et al. 2019; 2020).
340 Furthermore, molecular studies have allowed understanding the recombination and
341 linkage disequilibrium for genes located inside and outside of inversions (for example,
342 Schaeffer et al. 2003; Laayouni et al. 2003; Schaeffer and Anderson 2005; Pegueroles et
343 al. 2010, 2013, 2016; Stevison et al. 2011; Smukowski et al. 2015). However, we have
344 not completely identified all genetic elements (structural genes or regulatory sequences)
345 located inside inversions or outside but close to them. Neither and even more difficult,
346 we have not properly understood their possible interactions at functional level. Due to
347 all these shortcomings in our knowledge and also from an evolutionary point of view, it
348 is still fundamental to study the chromosomal inversions as whole genetic units
349 although the understanding of their molecular content is still incomplete (Pennisi 2017).

350 Although Font Groga and Avala are populations located in the central area of the
351 *D. subobscura* distribution (Krimbas 1992, 1993), they differ regarding historic,
352 climatic and biotic factors. For this reason, their inversion chromosomal polymorphisms
353 are distinct in term of composition and frequencies. This species presents a high
354 capacity of dispersion (Begon 1976; Serra et al. 1987; Ayala et al. 1989) and gene flow
355 (Araúz et al. 2009; Mestres et al. 2009; Pegueroles et al. 2013) that would tend to unify
356 the chromosomal composition between populations. Thus, given the particular
357 environmental conditions of each population, natural selection would likely have a great
358 effect on adaptive inversions generating the differentiation. However, exactly the same
359 thermal adapted inversions (‘cold adapted’: A_{st} , A_1 , J_{st} , U_{st} , E_{st} and O_{st} ; ‘warm adapted’:
360 A_2 , J_1 , U_{1+2} , U_{1+8+2} , E_{1+2+9} , $E_{1+2+9+12}$, O_{3+4} , O_{3+4+1} and O_{3+4+8}) were present in both
361 populations, Font Groga (Galludo et al. 2018) and Avala. In the latter, most of them (9
362 out of 15, after the FDR correction) were significant with PC1, PC2 or both, being these
363 principal components related mainly with temperature. These results differed from that

364 from Font Groga (Galludo et al. 2018), where 14 out of 15 inversion were significant,
365 but it is worth noting that the principal components in that population were not
366 equivalent to those from Avala. For instance, the PC1 from Font Groga depended
367 strongly of temperatures, but also of humidity and rainfall. Furthermore, in Avala
368 population, the relation of each inversion with the variation of one unit for a particular
369 environmental variable while maintaining constant the others, indicated that most
370 inversions (with the exceptions of the $E_{1+2+9+12}$ and O_{3+4+6} inversions) do not change
371 appreciably. This result could be a consequence of annual fluctuations of climatic
372 variables in Avala during the period 2014-2017, as no clear directional change was
373 present for any of the studied variables, no changes were detected for the thermal
374 adapted inversions.

375 However, it was relevant to study the change of chromosomal inversions over
376 time in Avala population (2004-2017). From a qualitative point of view, several
377 inversions considered warm and arid were detected recently for the first time and in low
378 frequencies in Avala (J_{3+4} in 2015 and U_{1+2+3} 2017). It could be explained by a tendency
379 towards decreased humidity in that region, and those inversions could have arrived from
380 the Eastern Mediterranean region. Instead, the quantitative study of inversion
381 chromosomal frequencies produced the main results. Thus, for all polymorphic
382 chromosomes of *D. subobscura*, the ‘cold’ adapted inversions remained rather constant
383 for the period 2004-2017. In a global warming scenario, a decrease in these frequencies
384 would be expected. A quite similar result was observed for the ‘warm’ inversions with a
385 notable exception, the U chromosome, showed a significant increase from 2004 to 2011
386 samples mainly due to the increase in frequency of U_{1+2} inversion (Zivanovic et al.
387 2015). This inversion was most abundant in the Caucasus and Iran regions (Krimbas
388 1993). However, from 2011 no significant variation in frequency has been observed for

389 'warm' inversions. The paradox was that the increase of U 'warm' inversions was at the
390 expense of 'non-thermal' adapted inversion, not at the 'cold' adapted as it could be
391 expected. For these reasons, the 'non-thermal' adapted inversions remained quite stable
392 over time with the exception of the U chromosome, with a remarkable decrease in the
393 U_{1+2+6} frequency from 2004-2011.

394 It would seem that the chromosomal inversion frequencies of *D. subobscura*
395 from Avala have reached a plateau of stability. This result could be an artifact due to the
396 few years studied, and because inversion polymorphism data were not available for each
397 one of the 13 years the involved in the analysis. Perhaps longer periods of time (about
398 20 to 30 years) would be necessary to detect notable changes in inversion frequencies,
399 as was carried out in several previous researches with this species (Orengo and Prevosti
400 1996; Solé et al. 2002; Balanyà et al. 2004, 2006; Orengo et al. 2016; Zivanovic et al.
401 2019). However, in intermediate periods of time (about 15 years), changes in this
402 polymorphism were detected and in the direction expected by global warming
403 (Rodríguez-Trelles and Rodríguez 1998; Zivanovic and Mestres 2011; Zivanovic et al.
404 2014b). Other hypotheses are possible, for instance the occurrence of episodes of
405 intense cold or heat waves in any of the years in which the study is framed (Schar and
406 Jendritzky 2004; Founda and Giannakopoulos 2009; Barriopedro et al. 2011; Stefanon
407 et al. 2012). It was observed that *D. subobscura* inversion polymorphism was able to
408 respond to these changes (Rodríguez-Trelles et al. 2013). However, these climatic
409 episodes could generate particular results that could be wrongly interpreted from the
410 perspective of climate change. This distortion due to particular climatic conditions
411 could also be mitigated if the studied period is large enough.

412 On the topic of inversion frequencies variation according to global warming,
413 there are still relevant questions to answer: is there a limit to the frequency increase of

414 warm adapted inversions in a population? If there is a threshold, are *D. subobscura*
415 populations close to it? Previous researches would indicate that the accumulation of
416 ‘warm’ adapted inversions is additive, with interactions representing a secondary role, if
417 any (Zivanovic et al 2016; Arenas et al. 2018). The thermal adaptation QTL mapping in
418 *D. melanogaster* would support this observation (Morgan and Mackay 2006; Norry et
419 al. 2008; Borda et al. 2018). Usually, all characters on which directional selection acts
420 have a limit, because other fitness-related characters are adversely affected (for a
421 revision see Lynch and Walsh 1998; Sgrò and Hoffmann 2004). For *D. subobscura*, the
422 ‘warm’ adapted inversions could contain genes or have genes in linkage disequilibrium
423 located near them, which would be dragged by the directional thermal selection
424 generating serious alterations of fitness. Moreover, it is worth to remember the complex
425 organization of chromosomal inversions in this species. From a historic point of view
426 and due to its large number of heterozygotes for inversions collected in natural
427 populations it was considered that *D. subobscura* could carry systems of balanced
428 lethals (Krimbas 1992, 1993 for a complete revision). Although inversions with
429 heterotic effect carrying lethal genes have been described in this species (Mestres et al.
430 2001), it was generally observed that the frequency of homozygotes and heterozygotes
431 for inversions fits panmictic expectations. Moreover, some arrangements constituted by
432 non-overlapped inversions located far away in the same chromosome were not broken
433 by recombination, likely due to linkage disequilibrium (Sperlich and Feuerbach 1969;
434 Sperlich and Feuerbach-Mravlag 1974; Mestres et al. 1998). If one of the inversions in
435 the arrangement contained the thermal adapted genes, all inversions constituting this
436 arrangement would be selected together. This selection would have consequences over
437 other fitness traits, and for this reason trade-offs would likely be necessary. Fitness

438 trade-offs involving inversions have been recently described in *D. melanogaster*
439 (Durmaz et al. 2018).

440 Is there any evidence indicating that any *D. subobscura* population has already
441 reached a ‘warm’ adapted inversions accumulation threshold? In a recent study in the *D.*
442 *subobscura* isolated Atlantic population of Madeira (Madrenas et al. 2020), the
443 frequencies of thermal adapted inversions and the *CTI* remained quite stable over time,
444 although there was a clear indication of global warming in the island (significant
445 increase of T_{min} , T_{max} and T_{mean}). The frequencies of the ‘cold’ adapted inversions
446 A_{st} (0.228) and E_{st} (0.257) were not negligible, indicating that they could contain genes
447 adaptive to other prominent environmental conditions for the species survival or the
448 inversion system of the species does not allow to continue with the directional selection.
449 Avala is not an isolated population and could change also due to gene flow, receiving
450 climatic adapted inversions. In this sense, it will be interesting to follow up the
451 evolution in frequency of J_{3+4} , U_{1+2+3} and O_{3+4+2} inversions, considered adaptive to
452 warm and dry conditions. Moreover, the *CTI* values estimated in this study (0.330 in
453 average) are not particularly high, and in this sense the composition of ‘warm’ adapted
454 inversions could increase, because the maximum value (0.958) was from the Italian
455 population of Etna (Arenas et al. 2018). Probably, the stability detected in Avala could
456 be a transient situation and longer periods of time should be studied in the future.
457 Furthermore, the inversion composition of Avala must be adaptive, not only regarding
458 the thermal variables, but also to other environmental conditions. In conclusion, it
459 would seem that depending on the environmental conditions, each *D. subobscura*
460 population could accumulate until a certain maximum amount of thermal adapted
461 inversions without altering other fitness components. Thus, the threshold for thermal

462 adapted inversions accumulation would probably be different in each population
463 depending on the particular environmental conditions of its ecosystem.

464 Finally, it is worth noting that natural ecosystems are complex in structure. The
465 climatic variables as temperatures, humidity and rainfall act changing the communities
466 and *D. subobscura* is an element of them. To study the direct effect of these variables
467 on the chromosomal inversion polymorphism is possibly a reduction and simplification
468 of the biological problem. Probably, inversions contain genes that could confer adaptive
469 advantage in front a combination of conditions, both physical and biological present in
470 the ecosystems (Galludo et al. 2018; Kapun and Flatt 2019). The interactions of genes
471 located in the chromosomal structure of *D. subobscura* karyotype would be responsible
472 for adaptations to a wide range of situations that take place in the ecosystems. All these
473 topics deserve future studies because the adaptive value of inversions to different
474 climatic conditions has been described in many species (Hoffmann et al. 2004; Ananina
475 et al. 2004; Etges and Levitan 2008; Takahashi and Takano-Shimizu 2011; van
476 Heerwaarden et al. 2012; Ayala et al. 2014; Berg et al. 2017, Kapun and Flatt 2019).

477

478 **Acknowledgements**

479 We dedicate this research to the memory of Prof. George W. Gilchrist (1954–
480 2020), an excellent evolutionary scientist and friend. This study was financially
481 supported by grants from the Ministry of Education, Science and Technological
482 Development of the Republic of Serbia (grant number 451-03-9/2021-14/200007), the
483 Ministerio de Economía y Competitividad, Spain (CTM2017-88080 AEI/FEDER, UE)
484 and the Generalitat de Catalunya, Spain (2017SGR 1120 and 2017SGR 622). The
485 authors declare that they have no conflict of interest.

486

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757

758

759 **FIGURE LEGENDS**

760 **Fig. 1** Multivariate analysis of the chromosomal inversion polymorphism from Avala
761 and Font Groga populations of *D. subobscura*. (A) Principal Coordinate Analysis. On
762 the left are grouped the samples from Avala and on the right those from Font Groga. (B)
763 GEVA-Ward cluster analysis. The first partition generates two groups, one with the
764 Avala samples (left) and the other with those from Font Groga (right). The
765 nomenclature used is: A14 (Avala 2014), A15 (Avala 2015), A16 (Avala 2016), A17
766 (Avala 2017), FG11 (Font Groga 2011), FG12 (Font Groga 2012), FG13 (Font Groga
767 2013), FG14 (Font Groga 2014) and FG15 (Font Groga 2015).

768 **Fig. 2** Variation in frequency (percentage) for the Avala chromosomal inversions
769 classified according to thermal adaptation in the years 2004, 2011, 2014, 2015, 2016
770 and 2017. (A) ‘Cold’ adapted inversions. (B) ‘Warm’ adapted inversions. (C). ‘Non-
771 thermal’ adapted inversions. The symbols stand for: black circle for A chromosome,
772 circle with a cross for J chromosome, white circle for U chromosome, inverted triangle
773 for E chromosome and diamond for O chromosome.

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