

1 **RATE OF CHANGE FOR THE THERMAL ADAPTED INVERSIONS IN**
2 ***DROSOPHILA SUBOBSCURA***

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20

21 **Abstract**

22 The changes of chromosomal inversion polymorphism composition of *Drosophila*
23 *subobscura* in samples from Apatin (Serbia) were studied in a 24-years interval (1994-
24 2018). The variation was significant for all autosomes and directional, increasing the
25 inversions considered as "warm" whereas those reported as "cold" decreased.
26 Furthermore, the Chromosomal Thermal Index (CTI), which allows studying the
27 thermal adaptation of the whole karyotype increased significantly in that period of time.
28 These results were in agreement with the indicators of global warming in Apatin: a
29 trend to increase of the mean, maximum and minimum (this latter even significant)
30 temperatures, and an erratic pattern of rainfall (also usual in global warming). The
31 deviations from the Wright-Fisher model of genetic drift were used to consider the
32 possible effect of migration or selection as evolutionary factors responsible for the
33 change in inversion frequencies. To quantify approximately the rate of change in the
34 frequencies, for each kind of inversions ("cold", "warm" and "non-thermal adapted"), the
35 difference in frequency between the Apatin samples obtained in 1994 and 2018 was
36 computed and then it was divided by the number of years elapsed. This rate was always
37 higher (from twice as many as thirty times more depending on the autosome) for
38 thermal adapted inversions ("cold" or "warm") than the "non-thermal" adapted. From this
39 study, it could be concluded that the chromosomal inversions of *D. subobscura* could
40 change (in composition and frequencies) in a predictable direction and a rather "rapid"
41 rhythm to adapt to the global warming scenario.

42

43 **Keywords** Chromosomal inversions, adaptation, selection, temperature, global
44 warming, evolutionary rate

45 **Introduction**

46 Chromosomal inversions were described for the first time more than one century ago
47 and their adaptive potential was demonstrated by Dobzhansky in a large number of
48 observations and experiments (Dobzhansky 1970), including his outstanding series of
49 papers on the Genetics of natural populations (the whole compilation can be found in
50 Lewontin et al. 1981). He and his team worked with *Drosophila pseudoobscura*, a
51 species distributed in the New World with a rich inversion polymorphism commonly
52 only in the third chromosome, although occasionally inversions in other chromosomes
53 have been observed (Powell 1992, 1997). At the same time in Europe, researchers who
54 worked in evolutionary genetics used another species of the same genus, *D. subobscura*.
55 In its karyotype, this species presented a large number of inversions and different
56 combinations of them in the same chromosome (the so-called arrangements, although
57 we will use only the word 'inversions' in this paper to simplify) (Krimbas 1992, 1993).
58 This karyotype is composed by four large acrocentric autosomes (named E, J, U and O),
59 the acrocentric sexual chromosome A (=X) and one dot, the only one without
60 inversions. The *D. subobscura* chromosomes A, E, J, U, O and dot correspond to the
61 Muller elements A, C, D, B, E and F, respectively (Powell 1997). In the Palearctic
62 region, the frequencies of thirteen inversions presented significant correlation with
63 latitude, and this result could be attributed to selective processes producing adaptations
64 or to historic events (Krimbas and Loukas 1980; Krimbas 1992). The American
65 colonization by *D. subobscura* (the species was first detected in 1978 in South America
66 and later in 1982 in North America, Prevosti et al. 1988) gave a clear answer to this
67 interesting question, the generation of latitudinal clines and in the same sense than those
68 observed in the Palearctic region, both in South and North America, in a short period of
69 time (in less than five years) strongly supported the hypothesis of selection as the main

70 mechanism producing these clines (Prevosti et al. 1985, 1988, 1989). Moreover in the
71 American continent, several inversions presented significant latitudinal clines although
72 they were associated with lethal genes (Mestres et al. 1990, 1992, 1995, 2001).
73 Although migration could be a response of the organisms to the environmental
74 conditions, only selection is adaptive. Therefore, natural selection was powerful and fast
75 producing this particular adaptive and geographical distribution of inversions in
76 America.

77 The chromosomal inversion polymorphism of *D. subobscura* proved also to be
78 adaptive to climatic change. Orengo and Prevosti (1996) pioneered this research trying
79 to answer this interesting biological question: what is the response of selection acting on
80 inversions in front to climatic change? Later, other investigators working with this
81 species confirmed their results and added more information to this fundamental and
82 very current issue (Rodríguez-Trelles and Rodríguez 1998; Solé et al. 2002; Balanyà et
83 al. 2004, 2006, 2009; Rezende et al. 2010; Orengo et al. 2016; Galludo et al. 2018). We
84 focused to analyze this evolutionary problem in another area of the Palearctic region,
85 the Balkans. It has the particularity that it is a relatively isolated zone by distinct
86 mountain ranges and is considered a refuge for animals and plants during the last
87 glaciation (Taberlet et al. 1998; Hewitt 1999, 2000; Heckel et al. 2005; Alexandri et al.
88 2012; Kindler et al. 2018). Therefore, these conditions would allow studying singular
89 patterns of evolution that could be different from those of other Palearctic areas. We
90 studied different aspects of the adaptation of chromosomal inversions to global warming
91 in distinct Balkan populations, such as the variations of inversion frequencies over time
92 (for each chromosome or for the whole karyotype using the *CTI* index), the influence of
93 several meteorological variables on these changes, the effect of inbreeding on thermal
94 adaptation, etc. (Zivanovic and Mestres 2010; Zivanovic et al. 2012, 2014a, 2014b,

95 2015, 2016; Arenas et al. 2018). However, one question still remained open on this
96 evolutionary topic: what is the rate of change in frequency of thermal adapted
97 inversions due to natural selection? To obtain an answer was the main goal of the
98 present research. For this purpose, we have analyzed again the inversion polymorphism
99 of the Serbian population of Apatin, previously studied in 1994 (Zivanovic et al. 2002),
100 to compare the changes in frequencies of both polymorphisms. Additionally, we had the
101 results from a small collection obtained in the years 2008 and 2009 (Zivanovic and
102 Mestres 2011), which allowed us to have a middle point in the 24 years elapsed between
103 1994 and 2018 and therefore to observe the variation pattern of inversions.

104 **Materials and Methods**

105 **Fly sampling and preparation of chromosomes**

106 *Drosophila subobscura* individuals from Apatin were collected on 18th to 20th June
107 2018 in the same place and month as the samples obtained in 1994, 2008 and 2009
108 (Zivanovic et al. 2002; Zivanovic and Mestres 2011). Flies were collected using 40
109 fermenting apple baits, from 6 a.m. to 8 a.m. and from 5 p.m. to 8 p.m. The sampling
110 site was a poplar (*Populus alba*) forest located in a swampy zone at the left bank of
111 Danube river (45°40'N, 19° 00'E), near the town of Apatin about 200 km NW of
112 Belgrade. This region is known as Upper Danube wetland area, which is included in the
113 Ramsar List (list of wetlands of international importance). It is usually flooded
114 producing a very humid habitat where the impact produced by the human being is small.
115 Samples from 2008 and 2009 were small because they were obtained in only one day
116 (Zivanovic and Mestres 2011). For this reason, they have been grouped and named
117 2008+2009.

118 Wild males and sons of wild females were crossed in individual vials with virgin
 119 females from the Kussnacht reference strain, which was homokaryotypic for the
 120 standard arrangements in all five chromosomes (A, E, J, U and O). Third instar larvae
 121 from the F₁ were dissected, and polytene chromosomes were stained and squashed in
 122 aceto-orcein solution. Chromosomal inversions were identified using the chromosomal
 123 maps of Kunze-Mühl and Müller (1958) and Krimbas (1993). To obtain the karyotypes
 124 with a probability higher than 0.99, at least eight larvae were analyzed from the progeny
 125 of each cross. All crosses were carried out at 18°C, 60% relative humidity and 12h/12h
 126 light/dark cycle. Finally, chromosomal inversions were classified as -Cø(cold adapted),
 127 -Wø(warm adapted) and -Nø(non-thermal adapted) following the criterion first
 128 introduced by Menozzi and Krimbas (1992), and verified by Rego et al. (2010) and
 129 Arenas et al. (2018).

130

131 Climatic information and statistical analysis

132 Meteorological data from Apatin (mean, maximum and minimum temperatures,
 133 insolation, and rainfall) were provided by the Serbian Republic Hydrometeorological
 134 Service. The information recorded for the period 1994-2018 was used in our analysis.
 135 For the different samples from Apatin, the Index of Free Recombination (IFR) proposed
 136 by Carson (1955) was used to estimate the degree of chromosomal inversion
 137 polymorphism and the Chromosomal Thermal Index (*CTI*) was computed to measure
 138 the thermal adaptation of the whole karyotype (Arenas et al. 2018). This index is
 139 defined by the expression:

140

$$CTI = \frac{W + C}{W + C + N}$$

141

where *W* , *C* and *N* represent the total number of -warmø -coldø and -thermally

142

adaptedøchromosomes (*N* = *W* + *C*). To test the hypothesis of equality in *CTI*

143 indexes a scored statistical test was also developed (for complete information on this
144 statistic and the associated test see Arenas et al. 2018).

145 Fisher's exact test was computed to compare the differences in chromosomal
146 inversion frequencies between samples from different years. This test was also used to
147 study the deviations from H-W expectations for the karyotypes obtained in 2018
148 collection. In all cases, the R function *fisher.test* was used and the corresponding *p*
149 values were obtained by means of a bootstrap (100,000 runs). In the case of multiple
150 comparisons, the FDR (False Discovery Rate) correction was applied according to
151 Benjamini and Hochberg (1995). In all analyses, *p*-values lower than 0.05 were
152 considered to be statistically significant.

153 Furthermore, to test whether the changes in the chromosomal inversions were
154 due to drift or to a deterministic factor (migration or selection) an analysis was carried
155 out using the Wright-Fisher model (Crow and Kimura 1970). According to it, the
156 expected mean frequency in generation *t*, \bar{p}_t , given the initial mean frequency p_0 is,

157
$$\bar{p}_t = p_0,$$

158 and the variance is estimated by,

159
$$V_t = p_0 q_0 \left[1 - \frac{p_0}{N} \left(1 - \frac{1}{N} \right)^t \right],$$

160 which depends on the initial frequency, the number of generations (*t*) and the effective
161 population size (*N*). In natural populations of *D. subobscura*, the number of generations
162 per year was estimated as five (Begon 1976; Mestres et al. 2001). With regard to *N*, for
163 Balkan and other central Palearctic populations in the peak of the species expansion (as
164 it is the case of the June samples from Apatin) a proper estimate would be 10,000
165 individuals (Begon 1977; Begon et al. 1980; Mestres and Serra 1991; Zivanovic et al.
166 2007; Araúz et al. 2009; Kurbalija-Novacic et al. 2013).

167 Finally, in order to have an approximate estimate, although not accurate, of the
168 rate of change in the frequencies of chromosomal inversions, we have simply
169 calculated, for each kind of inversions, the difference in frequency between the Apatin
170 samples obtained in 2018 and 1994, and then it was divided by the number of years
171 elapsed. Although it is a rough approximation it is useful for the aim of comparison
172 between the different chromosomes. The unit obtained for this procedure is the variation
173 in frequency *per year*.

174

175 **Results and Discussion**

176 Nowadays, there is evidence that unequivocally shows that climate change is a fact
177 (IPCC 2014) and a high consensus on the hypothesis of global warming exists among
178 most scientists studying this topic (Cook et al. 2016). This is considered one of the most
179 serious problems that affect our planet (Ripple et al. 2017). However, for analyzing
180 whether the chromosomal polymorphism from Apatin changed over time according to
181 global warming expectations, it is essential first to assess the magnitude of this climate
182 change in that location, that it is not under the direct influence of human activities
183 (Supplementary Table 1 and Figure 1). In Apatin, the temperatures recorded from 1994
184 to 2018 showed a trend to increase (T_{mean}: slope = 0.0405, t = 1.0396, *p*-value =
185 0.3093; T_{max}: slope = 0.0313, t = 0.6331, *p*-value = 0.5329) and it was even significant
186 for T_{min} (slope = 0.0736, t = 2.7506, *p*-value = 0.0114). Therefore, in only 24 years
187 temperatures increased, in agreement with global warming expectations. The insolation
188 remained rather stable over time (slope = 60.1012, t = 60.1014, *p*-value = 0.9201), and
189 rainfall showed an irregular pattern, also a common effect of global warming.

190 The chromosomal polymorphism obtained in the present sample from Apatin,
191 with those from previous collections, is presented in Table 1. From a descriptive point
192 of view, the warm adapted U_{1+8+2} , $E_{1+2+9+12}$ inversions began to be found in 2008+2009
193 collection and increased in frequency over time. The warm inversion O_{3+4+8} , was
194 detected for the first time in 2018. When comparing the chromosomal frequencies
195 between 1994 and 2018 and after carrying out the FDR correction, the differences in A
196 chromosome were not significant (p -value = 0.2637), but they were significant for the
197 others (J chromosome, p -value = 7.320e-05; U chromosome, p -value = 0.0124; E
198 chromosome, p -value = 0.0124; O chromosome, p -value = 0.0124). Considering all five
199 chromosomes together the difference in chromosomal composition is significant (p -
200 value = 0.0010). This comparison over years can also be computed considering only
201 cold and warm adapted inversions. Only the A chromosome was not significant (p -
202 value = 0.2927), whereas the other chromosomes were significant (J chromosome p -
203 value = 7.320e-05; U chromosome p -value = 1.823e-10; E chromosome p -value =
204 9.444e-15; O chromosome p -value = 1.4387e-06). To analyze whether these changes
205 could be attributed to a deterministic evolutionary factor (migration or selection) the
206 deviations from the Wright-Fisher model are presented in Table 2. Likely, three thermal
207 adapted inversions (U_{1+8+2} , $E_{1+2+9+12}$ and O_{3+4+8}) reached Apatin by migration, because it
208 is considered that inversion arisen only once in natural populations (Krimbas 1993;
209 Powell 1997). Moreover, migration and gene flow have been reported in *D. subobscura*
210 natural populations (Latorre et al. 1992; Krimbas 1993, Pascual et al. 2001; Zivanovic
211 et al. 2007; Pegueroles et al. 2013). When the computation was applicable, analyzing
212 the remaining thermal adapted inversions, out of 12 of them 10 presented a frequency
213 out of the interval defined by p_{exp} (the 2018 expected frequency) multiplied by two
214 times the standard deviation generated by the model. Thus, a deviation from the model

215 expectations is observed in most cases. Although migration has to be considered,
216 selection likely presents a preponderant role, because in all *D. subobscura* populations
217 so far analyzed (from both, Palearctic region and America) the inversions which change
218 over time are usually the same, and their changes in frequency are in the same direction
219 (Orengo and Prevosti 1996; Rodríguez-Trelles and Rodríguez 1998; Solé et al. 2002;
220 Balanyà et al. 2004, 2006, 2009; Zivanovic and Mestres 2010, 2011; Rezende et al.
221 2010; Zivanovic et al. 2012, 2014b, 2015; Orengo et al. 2016). Apatin population is not
222 an exception, and "warm" inversions increase in frequency, whereas "cold" decrease
223 (Table 1). A particular case was the A₁ inversion defined as "cold" adapted and
224 presenting a particular distribution from SE (high frequency) to NW (low frequency) in
225 the Palearctic region (Krimbas 1993). In this study, its frequency did not change over
226 time and did not deviate from the prediction of genetic drift model. Almost absence of
227 frequency change for this inversion was also detected in a five years study in Barcelona
228 (Galludo et al. 2018), indicating a particular behavior that deserves to be studied in
229 future researches. Finally, two inversions described as not related with thermal
230 adaptation (U₁₊₂₊₆ and E₈) also deviate from Wright-Fisher expectations. In the
231 Palearctic region, they showed a particular distribution. The U₁₊₂₊₆ is most frequent in
232 Greece, but decreasing its abundance in concentric circles towards the periphery. On the
233 other hand, the E₈ presents a similar pattern but centered in Anatolia, where it is most
234 abundant (Krimbas 1992, 1993).

235 The values for the *CTI* index were 0.347, 0.025 and 0.342 for the Apatin
236 samples of 1994, 2008+2009 and 2018, respectively. The differences were significant
237 when comparing 1994 vs. 2018 (test = 10.924; *p*-value = 1e-05), and 2008+2009 vs.
238 2018 (test = 3.865; *p*-value = 1e-04). These *CTI* results corroborate the change in
239 abundance of thermal adapted inversions in accordance with global warming and are in

240 agreement with results obtained from other Palearctic populations, including those from
241 the Balkans (Arenas et al. 2018).

242 The karyotypes observed in 2018, with those from previous collections in the
243 same population, are shown in Table 3. The 2018 sample did not present a significant
244 deviation from Hardy-Weinberg equilibrium for any of the chromosomes (J
245 chromosome p -value = 1; U chromosome p -value = 0.9703; E chromosome p -value =
246 0.9901; O chromosome p -value = 1) or when considering all of them together (p -value
247 = 1). Therefore, inversions seem to combine at random to constitute the genotypes of
248 the next generations. The IFR index decreased between 1994 and 2018 (84.62 ± 1.55
249 and 81.83 ± 0.91 , respectively), indicating a small increase in the amount of inversion
250 polymorphism (Krimbas 1992). Finally, when comparing the karyotypic composition
251 between 1994 and 2018, it changed significantly for all chromosomes (J chromosome,
252 p -value = 0.0040; U chromosome, p -value = 0.0099; E chromosome, p -value = 0.0099;
253 O chromosome, p -value = 0.0099) and also for the whole karyotype (p -value = 0.0010).
254 All these results are in agreement with previous studies from this species showing that
255 inversion chromosomal polymorphism changes according to global warming
256 expectations (Orengo and Prevosti 1996; Rodríguez-Trelles and Rodríguez 1998; Solé
257 et al. 2002; Balanyà et al. 2004, 2006, 2009; Rezende et al. 2010; Zivanovic and
258 Mestres 2011; Zivanovic et al. 2012, 2015; Orengo et al. 2016; Arenas et al. 2018).

259 In the field of biological evolution, there are some interesting questions that are
260 interrelated, how intense is the effect of natural selection and therefore what is the rate
261 of the evolutionary change? For quantitative traits, many measures were developed
262 (Haldane 1949; Gingerich 1983; Hendry and Kinnison 1999), but there are not for the
263 changes in inversion frequencies. Although it is a rather inaccurate measure, only for
264 the aim of comparison we have computed a simple rate of change for the inversion

265 frequencies. The results obtained when considering the inversions as ‘cold’ and ‘warm’ and
266 ‘non-thermal’ adapted are presented in Table 4. With the exception of the A
267 chromosome, which shows a particular behavior for this trait (Zivanovic et al. 2015;
268 Galludo et al. 2018), all thermal adapted inversions (‘cold’ or ‘warm’) from the other
269 chromosomes (J, U, E and O) changed in a rather similar rate (considering the absolute
270 value), but higher than the ‘non-thermal’ adapted. In particular and considering the
271 absolute values for the U, E and O chromosomes, it is interesting the comparison by
272 simply dividing the rate of ‘cold’ or ‘warm’ adapted inversion by that from the ‘non-
273 thermal’ adapted. For the U chromosome the values obtained are 3.9974 and 2.9872,
274 when using the ‘cold’ and ‘warm’ adapted inversions, respectively. The ratio of the
275 division was not so large for the E chromosome (3.1229 and 2.1244, respectively), and
276 the largest values were obtained for the O chromosome (33.4474 and 32.4474).
277 Moreover, using the small sample from 2008+2009 as an approximate middle point of
278 the whole period studied (Table 4), it is possible to observe that depending on the
279 chromosome, the rhythm of change of the thermal adapted inversions (‘cold’ or ‘warm’)
280 is first ‘fast’ and later ‘slow’ (U chromosome) or vice versa (J and E chromosomes) or
281 even rather constant (O chromosome). Likely, the small and random fluctuations
282 observed for the ‘non-thermal’ adapted inversions would be due to genetic drift.

283 When considering microevolution (in the scale of short periods of years), the
284 inversion chromosomal polymorphism seems to react very fast to changes in the
285 environmental conditions. The supergenes present at this level of genetic variability
286 could be an excellent and available substrate on which natural selection would easily
287 act. In *D. subobscura* for instance, significant changes in frequency for the U
288 chromosomal inversions according to global warming expectations were reported on a
289 period of only seven years (Zivanovic et al. 2015). Significant variations were

290 commonly observed in periods of 20 to 30 years, in both Palearctic and American
291 populations (Solé et al. 2002, Balanyà et al. 2004, 2006, 2009). Another outstanding
292 observation was the rapid formation (in few years) of latitudinal clines in the North and
293 South American regions colonized by this species, and in the same sense that those
294 described in the Palearctic region long time ago (reviewed in Prevosti et al. 1988, 1989).
295 However, this was not the case for quantitative traits in the same species, which seemed
296 to respond slower than chromosomal inversions to the action of natural selection
297 (Pegueroles et al. 1995; Huey et al. 2000; Gilchrist et al. 2001). Probably, their genetic
298 architecture and the distinct relation between genotype and phenotype would be the
299 responsible of this different adaptive behavior.

300 In summary our analysis, although preliminary, gives new insights in the
301 knowledge of the potentiality of organisms to adapt to the general problem of global
302 warming. The chromosomal inversion polymorphism of *D. subobscura* is able to
303 change over time (in composition and frequencies) in front of this alteration of the
304 environment. Furthermore, this shift is rather predictable, because we could know quite
305 accurately which inversions would be involved, in which direction and in which rate
306 (rapidly) would change to adapt to the global warming scenario. As a conclusion, this
307 research provides valuable information on the adaptive capacity of organisms given the
308 anthropogenic activities responsible of the climate change in our World.

309

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317

318 **Compliance with ethical standards**

319 **Conflict of interest.** The authors declare that they have no conflict of interest.

320

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489 **Table 1** Frequencies of chromosomal arrangements of *D. subobscura* in Apatin for the
 490 month of June from three different periods (1994, 2008+2009 and 2018).

491

Chrom. arrangement	Thermal adapt.	1994		2008+2009		2018	
		n	%	n	%	n	%
A _{st}	C	25	50.0	10	50.0	34	37.8
A ₁	C	21	42.0	9	45.0	42	46.7
A ₂	W	4	8.0	1	5.0	14	15.5
Total		50		20		90	
J _{st}	C	39	39.0	15	37.5	27	15.0
J ₁	W	61	61.0	25	62.5	153	85.0
Total		100		40		180	
U _{st}	C	52	52.0	11	27.5	26	14.4
U ₁	N	1	1.0	0	0	0	0
U ₁₊₂	W	23	23.0	19	47.5	77	42.8
U ₁₊₂₊₆	N	24	24.0	7	17.5	62	34.4
U ₁₊₈₊₂	W	0	0	3	7.5	15	8.3
Total		100		40		180	

E _{st}	C	70	70.0	25	62.5	36	20.0
E ₁₊₂	N	2	2.0	0	0	12	6.7
E ₁₊₂₊₉	W	11	11.0	6	15.0	65	36.1
E ₁₊₂₊₉₊₁₂	W	0	0	2	5.0	16	8.9
E ₈	N	17	17.0	7	17.5	51	28.3
Total		100		40		180	

O _{st}	C	61	61.0	16	40.0	55	30.5
O ₆	N	6	6.0	0	0	0	0
O ₂₂	N	0	0	1	2.5	0	0
O ₃₊₄	W	23	23.0	16	40.0	83	46.1
O ₃₊₄₊₁	W	8	8.0	2	5.0	21	11.7
O ₃₊₄₊₂	N	2	2.0	0	0	0	0
O ₃₊₄₊₆	N	0	0	0	0	8	4.4
O ₃₊₄₊₈	W	0	0	0	0	5	2.8
O ₃₊₄₊₂₂	N	0	0	5	12.5	8	4.4
Total		100		40		180	

492 n = number of chromosomes.

493 With regard to thermal adaptation ‘C’ ‘W’ and ‘N’ stand for ‘cold’, ‘warm’ and ‘non-
494 thermal’ adapted, respectively. This classification was obtained following the criterion
495 of Menozzi and Krimbas (1992) and Rego et al. (2010).

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497

498 **Table 2** The Fisher-Wright expectations for the *D. subobscura* inversion chromosomal
 499 frequencies from Apatin. The variables are: p_0 (initial frequencies observed in 1994),
 500 p_{exp} (expected frequencies in 2018 according to the model) and p_{obs} (observed
 501 frequencies in 2018). *SD* stands for standard deviation calculated according to the
 502 model. The values of p_{obs} are presented in bold when they are not included in the
 503 interval $p_{exp} \pm 2*SD$.

504

Chrom. arrang.	Thermal adapt.	p_0	p_{exp}	$2*SD$	$p_{exp} \pm 2*SD$	p_{obs}
A _{st}	C	0.50	0.50	0.08	[0.42, 0.58]	0.38
A ₁	C	0.42	0.42	0.08	[0.34, 0.50]	0.47
A ₂	W	0.08	0.08	0.04	[0.04, 0.12]	0.16
J _{st}	C	0.39	0.39	0.08	[0.31, 0.47]	0.15
J ₁	W	0.61	0.61	0.08	[0.53, 0.69]	0.85
U _{st}	C	0.52	0.52	0.08	[0.44, 0.60]	0.14
U ₁	N	0.01	0.01	0.02	[-0.01, 0.03]	0.00
U ₁₊₂	W	0.23	0.23	0.07	[0.16, 0.30]	0.43
U ₁₊₂₊₆	N	0.24	0.24	0.07	[0.17, 0.31]	0.34
U ₁₊₈₊₂	W	0.00	0.00	N/A	N/A	0.08
E _{st}	C	0.70	0.70	0.07	[0.63, 0.77]	0.20
E ₁₊₂	N	0.02	0.02	0.02	[0.00, 0.04]	0.07
E ₁₊₂₊₉	W	0.11	0.11	0.05	[0.06, 0.16]	0.36
E ₁₊₂₊₉₊₁₂	W	0.00	0.00	N/A	N/A	0.09
E ₈	N	0.17	0.17	0.06	[0.11, 0.23]	0.28

O_{st}	C	0.61	0.61	0.08	[0.53, 0.69]	0.31
O_6	N	0.06	0.06	0.04	[0.02, 0.10]	0.00
O_{22}	N	0.00	0.00	N/A	N/A	0.00
$O_{\underline{3+4}}$	W	0.23	0.23	0.07	[0.16, 0.30]	0.46
$O_{\underline{3+4+1}}$	W	0.08	0.08	0.04	[0.04, 0.12]	0.12
$O_{\underline{3+4+2}}$	N	0.02	0.02	0.02	[0.00, 0.04]	0.00
$O_{\underline{3+4+6}}$	N	0.00	0.00	N/A	N/A	0.04
$O_{\underline{3+4+8}}$	W	0.00	0.00	N/A	N/A	0.03
$O_{\underline{3+4+22}}$	N	0.00	0.00	N/A	N/A	0.04

505

506 With regard to thermal adaptation $\neg C\emptyset$, $\neg W\emptyset$ and $\neg N\emptyset$ stand for \neg cold \emptyset , \neg warm \emptyset and \neg non-
507 thermal \emptyset adapted, respectively.

508 N/A = not applicable.

509

510

511 **Table 3** Frequencies of chromosomal karyotypes of *D. subobscura* in Apatin for the
 512 month of June from three different years (1994, 2008+2009 and 2018).

513

Karyotypes	1994		2008+2009		2018	
	n	%	n	%	n	%
J_{st}/J_{st}	9	18.0	2	10.0	2	2.2
J_{st}/J_1	21	42.0	11	55.0	23	25.5
J_1/J_1	20	40.0	7	35.0	65	72.2
Total	50		20		90	
U_{st}/U_{st}	17	34.0	0	0	4	4.4
$U_{st}/U_{\underline{1+2}}$	10	20.0	7	35.0	10	11.1
$U_{st}/U_{\underline{1+2+6}}$	8	16.0	2	10.0	6	6.7
$U_{st}/U_{\underline{1+8+2}}$	0	0	2	10.0	2	2.2
$U_1/U_{\underline{1+2}}$	1	2.0	0	0	0	0
$U_{\underline{1+2}}/U_{\underline{1+2}}$	3	6.0	4	20.0	19	21.1
$U_{\underline{1+2}}/U_{\underline{1+2+6}}$	6	12.0	3	15.0	23	25.5
$U_{\underline{1+2}}/U_{\underline{1+8+2}}$	0	0	1	5.0	6	6.7
$U_{\underline{1+2+6}}/U_{\underline{1+2+6}}$	5	10.0	1	5.0	13	14.4
$U_{\underline{1+2+6}}/U_{\underline{1+8+2}}$	0	0	0	0	7	7.8
Total	50		20		90	

E_{st}/E_{st}	29	58.0	6	30.0	8	8.9
E_{st}/E_{1+2}	1	2.0	0	0	1	1.1
E_{st}/E_{1+2+9}	8	16.0	6	30.0	9	10.0
$E_{st}/E_{1+2+9+12}$	0	0	2	10.0	1	1.1
E_{st}/E_8	3	6.0	5	25.0	9	10.0
E_{1+2}/E_{1+2+9}	0	0	0	0	4	4.4
E_{1+2}/E_8	1	2.0	0	0	7	7.8
E_{1+2+9}/E_{1+2+9}	2	4.0	0	0	14	15.5
$E_{1+2+9}/E_{1+2+9+12}$	0	0	0	0	9	10.0
E_{1+2+9}/E_8	5	10.0	0	0	15	16.7
$E_{1+2+9+12}/E_{1+2+9+12}$	0	0	0	0	1	1.1
$E_{1+2+9+12}/E_8$	0	0	0	0	4	4.4
E_8/E_8	1	2.0	1	5.0	8	8.9
Total	50		20		90	

O_{st}/O_{st}	22	44.0	2	10.0	9	10.0
O_{st}/O_6	4	8.0	0	0	0	0
O_{st}/O_{22}	0	0	1	5.0	0	0
O_{st}/O_{3+4}	7	14.0	9	45.0	23	25.5
O_{st}/O_{3+4+1}	4	8.0	1	5.0	7	7.8
O_{st}/O_{3+4+2}	2	4.0	0	0	0	0
O_{st}/O_{3+4+6}	0	0	0	0	1	1.1
O_{st}/O_{3+4+8}	0	0	0	0	3	3.3
O_{st}/O_{3+4+22}	0	0	1	5.0	3	3.3
O_6/O_{3+4}	1	2.0	0	0	0	0

$O_6/O_{\underline{3+4+1}}$	1	2.0	0	0	0	0
$O_{\underline{3+4}}/O_{\underline{3+4}}$	7	14.0	2	10.0	19	21.1
$O_{\underline{3+4}}/O_{\underline{3+4+1}}$	1	2.0	0	0	12	13.3
$O_{\underline{3+4}}/O_{\underline{3+4+6}}$	0	0	0	0	4	4.4
$O_{\underline{3+4}}/O_{\underline{3+4+8}}$	0	0	0	0	2	2.2
$O_{\underline{3+4}}/O_{\underline{3+4+22}}$	0	0	3	15.0	4	4.4
$O_{\underline{3+4+1}}/O_{\underline{3+4+1}}$	1	2.0	0	0	0	0
$O_{\underline{3+4+1}}/O_{\underline{3+4+6}}$	0	0	0	0	2	2.2
$O_{\underline{3+4+1}}/O_{\underline{3+4+22}}$	0	0	1	5.0	0	0
$O_{\underline{3+4+6}}/O_{\underline{3+4+22}}$	0	0	0	0	1	1.1
Total	50		20		90	

514 n = number of chromosomes.

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516

517 **Table 4** Variation in frequency for the -cold-, -warm- and -non-thermal- inversions and
 518 arrangements of *D. subobscura* in the June samples of 1994, 2008+2009 and 2018 from
 519 Apatin. In the last column, the rate of change is presented.

Chromosomes	1994	2008+2009	2018	<i>Rate</i>
A Cold	0.920	0.950	0.845	60.00313
A Warm	0.080	0.059	0.156	0.00317
J Cold	0.390	0.375	0.150	60.01000
J Warm	0.610	0.625	0.850	0.01000
U Cold	0.520	0.275	0.144	60.01567
U Warm	0.230	0.550	0.511	0.01171
U Non-thermal	0.250	0.175	0.344	0.00392
E Cold	0.700	0.625	0.200	60.02083
E Warm	0.110	0.200	0.450	0.01417
E Non-thermal	0.190	0.175	0.350	0.00667
O Cold	0.610	0.400	0.305	60.01271
O Warm	0.310	0.450	0.606	0.01233
O Non-thermal	0.080	0.150	0.089	0.00038

520 *Rate* stands for the difference in frequency between the samples obtained in 2018 and
 521 1994, and then divided by the number of years elapsed.

522 Chromosomal inversions were classified as ~~−Cold~~, ~~−Warm~~ and ~~−Non-thermal~~ (non-
523 thermal adapted) following the criterion of Menozzi and Krimbas (1992) and Rego et al.
524 (2010).

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