1 **INVERSION POLYMORPHISM IN TWO SERBIAN NATURAL**

2 **POPULATIONS OF *Drosophila subobscura*: ANALYSIS OF**

3 **LONG-TERM CHANGES**

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21 **Running title**: Long-term changes in karyotypes

22

23   To study whether inversions (or arrangements) by themselves or karyotypes are the

24   global warming adaptive target of natural selection, two *Drosophila subobscura* Serbian

25   populations (Apatin and Petnica) were re-analyzed using different statistical approaches.

26   Both populations were sampled in an approximately 15 years period: Apatin in 1994

27   and 2008+2009 and Petnica in 1995 and 2010. For all chromosomes, the four

28   collections studied were in Hardy-Weinberg equilibrium. Thus, it seemed that

29   inversions (or arrangements) combined at random to constitute populationsøkaryotypes.

30   However, there were differences in karyotypic frequencies along the years, although

31   they were significant only for Apatin population. Thus, inversions (or arrangements) are

32   likely the target of natural selection, because they presented long-term changes, but

33   combine at random to generate the corresponding karyotypic combinations.

34   In *Drosophila* genus, the chromosomal inversion polymorphism seems to be

35   adaptive and it is subject to strong selection, because their frequencies change in time.

36   For instance, short- (seasonal variation) and long-term changes (according to

37   environmental variations) were reported in different species of this genus [1-5]. In this

38   context, studies in the model species *Drosophila subobscura*, due to its rich

39   chromosomal polymorphism for inversions, gave new insights on this adaptive process

40   and the role of natural selection. Seasonal variation in chromosomal polymorphism

41   frequencies was observed and interpreted as an adaptive process [6-9]. Furthermore,

42   variations in the chromosomal polymorphism in time (long-term changes) were

43   considered a key element to monitor the global climate change (for a review see [10].

44   The role of natural selection was also observed in the latitudinal clinal variation of the

45   inversion frequencies, both in Palearctic and American colonizing populations [11-14].

46   Finally, in American populations of *D. subobscura*, the effect of natural selection on

47   several inversions (O_5 and O_{3+4+7}) could be measured [15].

48   However, although short- and long-term changes in the composition and

49   frequencies for chromosomal inversions or arrangements (overlapped inversions) have

50   been intensively analyzed, this is not the case with regard to inversion karyotypes. Few

51   studies have been carried out and limited information has been obtained [9, 16-20].

52   Inversions on one chromosome could not act independently, because the genome is an

53   integrated functional system. The genetic information carried by both homologous

54   chromosomes could have an important effect on the adaptive capacity. For instance,

55   some inversions (or arrangements) in one homologous chromosome combined with

56   those of the other homologous of the pair could provide a better adaptation to certain

57   environmental or climatic conditions. For this reason, the information provided by

58   karyotypes could generate new insights in the adaptive changes along time. Our aim has

59   been to re-analyze -using different statistical approaches- data on chromosomal

60   karyotypes from two Serbian populations, which were sampled two times each one in a

61   15 years period and to study the variation in their karyotypic frequencies.

62

63   MATERIAL AND METHODS

64   We have re-analyzed data from a couple of Serbian populations: Apatin

65   (sampled in 1994 and 2008+2009) and Petnica (collected in 1995 and 2010). Detailed

66   information regarding both populations can be found in [19] and [20]. Samples of

67   different years were strictly collected in the same place, month and equivalent day.

68   Males, and in some collections sons of wild females to increase the sample size, were

69   crossed individually with virgin females of the Kussnacht strain that were

70   homokaryotypic for standard chromosomal arrangements in all five chromosomes (A

71   (X), E, J, U and O). Once dissected from third instar larvae, polytene chromosomes

72   were stained and squashed in aceto-orcein solution. At least eight larvae from the

73   progeny of each cross were examined in order to know the inversion pattern of both

74   homologous chromosomes with a probability higher than 0.99. The chromosomal map

75   of Kunze-Mühl and Müller [21] and Krimbas [22] was used for cytological analysis of

76   the chromosomal inversions and arrangements and their nomenclature that of Kunze-

77   Mühl and Sperlich [23]. Departure of chromosomal karyotypes from Hardy óWeinberg

78   equilibrium and comparisons between samples were analyzed using Fisherøs exact test

79   (statistically significant p-$value$ < 0.05), as it is considered the best procedure in the

80   case of multiple alleles per locus [24], in our case, different inversions (or

81   arrangements) per chromosome. The corresponding p-$values$ were obtained using the

82   bootstrap procedure (100000 runs). These computations were carried out with R

83   packages (http://CRAN.R-project.org). Confidence intervals (CI) of karyotypic

84   frequencies were estimated according to the binomial distribution.

85   .

86   RESULTS

87   The observed and expected frequencies of chromosomal karyotypes are

88   presented in Table 1 (Apatin) and Table 2 (Petnica). With regard to the Apatin

89   population (1994), all chromosomes were in H-W equilibrium: J (*p-value* = 0.8956), U

90   (*p-value* = 0.8892), E (*p-value* = 0.4909) and O (*p-value* = 0.6626). For the same

91   population, but analyzing the 2008 + 2009 sample, for all chromosomes not significant

92   deviations from H-W equilibrium were detected: J (*p-value* = 0.8294), U (*p-value* =

93   0.9558), E (*p-value* = 0.9059) and O (*p-value* = 0.9288). In the case of Petnica

94   population, for the sample of 1995 all chromosomes were in H-W equilibrium: J (*p-

95   value* = 0.8973), U (*p-value* = 0.9311), E (*p-value* = 0.9967) and O (*p-value* = 0.8980).

96   Finally, for the same population, but sampled in 2010, H-W equilibrium was observed

97   for all chromosomes: J (*p-value* = 1), U (*p-value* = 1), E (*p-value* = 0.9337) and O (*p-

98   value* = 0.6089).

99   The comparisons between the karyotypic frequencies of both samples of Apatin

100 (1994 and 2008+2009) are presented in Fig. 1 (1A, J chromosome; 1B, U chromosome;

101 1C, E chromosome; 1D, O chromosome). With the exception of J chromosome, in all

102 chromosomes there were significant differences for karyotypic frequencies: J (*p-value* =

103 0.6376), U (*p-value* = 0.0026), E (*p-value* = 0.0148) and O (*p-value* = 0.0007). In

104 2008+2009 sample, karyotypes containing the arrangements U_{1+2} and U_{1+8+2} increased

105 in frequencies, whereas karyotypes with U_{st} tended to decrease, except U_{st}/ U_{1+2} and $U_{st}/$

106 U_{1+8+2} combinations (Table 1 and Fig. 1B). For the E chromosome (Table 1 and Fig.

107 1C), the E_{st}/E_{st} karyotype decreased, but it was an increase of E_{st}/E_{1+2+9}, E_{st}/E_8 and

                                                                                            5

108 $E_{st}/E_{1+2+9+12}$ (not present in 1994). Interestingly, it was a dramatic decrease of O_{st}/O_{st}

109 karyotype and a substantial increase of O_{st}/O_{3+4} (Table 1 and Fig. 1D). In both cases, CI

110 were almost non-overlapped. Many karyotypes presented in low frequencies in 1994

111 were missing in 2008+2009 (O_{st}/O_6, O_{st}/O_{3+4+2}, O_6/O_{3+4}, O_6/O_{3+4+1}, O_{3+4}/O_{3+4+1} and

112 O_{3+4+1}/O_{3+4+1}), whereas others were detected for the first time (O_{st}/O_{22}, O_{st}/O_{3+4+22} and

113 O_{3+4+1}/O_{3+4+22}). The O_{3+4} inversion increased in frequency (from 0.23 to 0.40), but not

114 the frequency of O_{3+4}/O_{3+4} karyotype, which decreased.

115   The karyotypic frequencies of Petnica samples (1995 and 2010) are graphically

116 shown in Fig. 2 (2A, J chromosome; 2B, U chromosome; 2C, E chromosome; 2D, O

117 chromosome). In this case, there were not significant frequency differences for any

118 chromosome: J (*p-value* = 0.3218), U (*p-value* = 0.0682), E (*p-value* = 0.0879) and O

119 (*p-value* = 0.0624). However, the differences for the U and O chromosomes were rather

120 close to significance. In 2010 collection, J_1/J_1 increased and J_{st}/J_{st} disappeared (Table 2

121 and Fig. 2A). With regard to the U chromosome (Table 2 and Fig. 2B), a small increase

122 in the U_{1+2}/U_{1+2} was observed, and karyotypes U_{1+2}/U_{1+8+2} and U_{1+2+6}/U_{1+8+2} were not

123 present in 1995. On the contrary, the frequency of homokaryotype U_{1+2+6}/U_{1+2+6}

124 homokaryotype decreased. The frequency of E_{st}/E_{st} karyotype decreased (Table 2 and

125 Fig. 2C), but E_{st}/E_{1+2+9} and E_{st}/E_8 also decreased in frequency (contrary to that found in

126 Apatin). As in Apatin, $E_{st}/E_{1+2+9+12}$ appeared for the first time in the 2010 collection.

127 Several karyotypes found in 1995 were not found later (E_{1+2}/E_{1+2+9} and E_{1+2}/E_8),

128 whereas $E_8/E_{1+2+9+12}$ appeared in the second collection. Also as in Apatin populations,

129 the frequency of O_{st}/O_{st} karyotype decreased in time (Table 2 and Fig. 2D). However,

130 O_{3+4}/O_{3+4} also decreased in frequency and that of O_{st}/O_{3+4} remained without variation.

131 Many karyotypic combinations disappeared in 2010 sample (O_{st}/O_{22}, O_6/O_{3+4+1},

132 O_{3+4}/O_{3+4+2}, O_{3+4+1}/O_{3+4+2} and O_{3+4+22}/O_{3+4+22}), but it was possible to find for the first

133   time O_{3+4}/O_{3+4+8}. Finally, other karyotypes increased their frequencies (O_{3+4}/O_{3+4+22} and

134   O_{3+4+1}/O_{3+4+22}).

135

136   DISCUSSION

137   If a genetic marker is in H-W equilibrium, a random combination of alleles to

138   constitute the next generation genotypes has to be assumed. In this situation, no relevant

139   effect of selection (and other evolutionary forces) seems to act on the studied genetic

140   marker. In our case, we focused in combinations of karyotypes, and for this reason to

141   find H-W equilibrium implies that gene inversions or arrangements combine at random

142   (or almost at random) to form the corresponding karyotypes. In Apatin population and

143   for 1994 and 2008+2009 samples, all chromosomes were in H-W equilibrium. Thus, it

144   seems that there is not a detectable effect of selection in Apatin. A similar situation was

145   found in Petnica: all chromosomes in both samples (1995 and 2010) were also in H-W

146   equilibrium. As in the population of Apatin, the effect of selection at this level seems

147   very low. In general, other researchers reported similar results in distinct *D. subobscura*

148   populations [16, 27-31]. However, several authors found several deviations from H-W

149   equilibrium for particular chromosomes [28, 29, 32].

150   The situation is different when the same population is compared using samples

151   collected in different years. In Apatin, significant changes in the frequencies of

152   karyotypes have been found for the U, E and O chromosomes. The most interesting

153   result is that inversion and karyotype frequencies have been changed in this 15 years

154   period, but inversions (or arrangements) combine at random to produce the karyotypes

155   (no H-W deviations). Thus, it seems that inversions *per se* are the target of selection,

156   not the karyotypic combinations produced. In *D. subobscura*, long-term changes of

157   inversions according to global warming expectations have been documented in both,

158 autochthonous and colonizing populations [10, 17-20, 25, 26, 33-35]. A similar result

159 was obtained for Petnica population. However, the differences in karyotypic frequencies

160 were not significant. Thus, the interactions of inversions located in the pair of

161 homologous chromosomes seem not to be the target of selection. Inversions or their

162 combinations (arrangements) are important by themselves and are directly under the

163 effect of natural selection. For this reason, it is correct to use the terminology õwarmö

164 and õcoldö adapted inversions. For instance, O_{st}, E_{st}, J_{st} or U_{st} can be considered õcoldö

165 adapted due their genetic content, with independence of other inversions in their

166 homologous chromosomes. However, in *D. subobscura* several examples of heterotic

167 effect of inversions have been reported. For instance, in American colonizing

168 populations the O_5 inversions presents a heterotic effect [15], but it is due to its

169 complete association with a lethal gene [36-38]. For this reason, the karyotype O_5/O_5

170 does not exist in American populations. A heterotic effect for an O_{3+4+7} arrangement

171 associated to a lethal gene was also described in the Californian population of Gilroy

172 [15]. This arrangement also presented a heterotic effect in crosses carried out in

173 laboratory conditions [39]. However, although it is a species with a rich chromosomal

174 polymorphism, *D. subobscura* is considered not a system of balanced strain.

175 Furthermore, it is accepted in general that homozygotes for gene arrangements are

176 found in the expected panmictic frequencies [22].

177   However, our study presents a number of limitations. To properly study the

178 karyotypic frequencies a large sample size is needed, because many inversion (or

179 arrangement) combinations exist producing different kind of karyotypes. In most

180 populations, many karyotypes will be present in low frequencies, thus non-negligible

181 sample sizes are needed to obtain accurate estimates of their frequencies. However, to

182 obtain karyotypic frequencies implies much more laboratory work than to only analyze

183 chromosomal polymorphism frequencies. Moreover, if the aim is to study the long-term

184 changes in karyotype composition it must collect exactly at the same site, month and

185 day (a correction is needed, because, according to Menzel et al. [40], spring/summer has

186 advanced 2.5 days per decade in Europe). Thus, the number of flies obtained in the

187 second period collection will depend on the particular conditions of the trapping day

188 [19]. Another limitation is that only combinations of inversions (or arrangements) from

189 the same pair of homologous chromosomes have been analyzed. Likely, the interactions

190 of combinations between inversions located in non-homologous chromosomes would be

191 an interesting topic, but an enormous sample size and laboratory work would be needed

192 due to the large number of possible combinations between different inversions of

193 distinct chromosomes.

194   In summary, our four samples analyzed (Apatin 1994, Apatin 2008+2009,

195 Petnica 1995 and Petnica 2010) were in H-W equilibrium. Thus, inversions (or

196 arrangements) seem to combine at random to form karyotypes. However, kayotypes of

197 both populations have changed along time (significant in Apatin and non-significant in

198 Petnica). Likely, inversions (or arrangements) have been under selection for being

199 adaptive to climatic changes, but they combine at random to constitute the karyotypes.

200 From our data, inversions (or arrangements) appear as the key elements being under

201 selection in the global warming environment.

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FIGURE LEGENDS:

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365 **Fig. 1.** Graphical representation of karyotypic frequencies obtained in Apatin, in 1994

366 (white) and 2008+2009 (grey). CI for each frequency is also presented. **A)** J

367 chromosome. **B)** U chromosome. **C)** E chromosome. **D)** O chromosome, where, a:

368 O_{st}/O_{st}; b: O_{st}/O_6; c: O_{st}/O_{22}; d: O_{st}/O_{3+4}; e: O_{st}/O_{3+4+1}; f: O_{st}/O_{3+4+2}; g: O_{st}/O_{3+4+22}; h:

369 O_6/O_{3+4}; i: O_6/O_{3+4+1}; j: O_{3+4}/O_{3+4}; k: O_{3+4}/O_{3+4+1}; l: O_{3+4}/O_{3+4+22}; m: O_{3+4+1}/O_{3+4+1}; n:

370 O_{3+4+1}/O_{3+4+22}.

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372 **Fig. 2.** Graphical representation of karyotypic frequencies obtained in Petnica, in 1995

373 (white) and 2010 (grey). CI for each frequency is also presented. **A)** J chromosome. **B)**

374 U chromosome. **C)** E chromosome. **D)** O chromosome, where, a: O_{st}/O_{st}; b: O_{st}/O_{22}; c:

375 O_{st}/O_{3+4}; d: O_{st}/O_{3+4+1}; e: O_{st}/O_{3+4+2}; f: O_6/O_{3+4+1}; g: O_{3+4}/O_{3+4}; h: O_{3+4}/O_{3+4+1}; i:

376 O_{3+4}/O_{3+4+2}; j: O_{3+4}/O_{3+4+8}; k: O_{3+4}/O_{3+4+22}; l: O_{3+4+1}/O_{3+4+1}; m: O_{3+4+1}/O_{3+4+2}; n:

377 O_{3+4+1}/O_{3+4+22}; o: O_{3+4+22}/O_{3+4+22}.

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380 **Table 1.** Frequencies (in percentage) of the observed (Obs.) and expected (Exp.)

381 karyotypes in both collections (1994 and 2008+2009) from Apatin population.

	1994			2008+2009	
	OBS.	EXP.		OBS.	EXP.
Karyotype	%	%		%	%
J_{st}/J_{st}	18.0	15.21		10.0	14.06
J_{st}/J_1	42.0	47.58		55.0	46.88
J_1/J_1	40.0	37.21		35.0	39.06
n	50	50		20	20
U_{st}/U_{st}	34.0	27.04		/	7.65
U_{st}/U_{1+2}	20.0	23.92		35.0	26.13
U_{st}/U_{1+2+6}	16.0	24.96		10.0	9.63

U_{st}/U_{1+8+2}	/	/		10.0	4.13
U_1/U_{1+2}	2.0	0.46		/	/
U_{1+2}/U_{1+2}	6.0	5.29		20.0	22.56
U_{1+2}/U_{1+2+6}	12.0	11.04		15.0	16.63
U_{1+2}/ U_{1+8+2}	/	/		5.0	7.13
U_{1+2+6}/U_{1+2+6}	10.0	5.76		5.0	3.06
Other		1.53			3.08
n	50	50		20	20
E_{st}/E_{st}	58.0	49.0		30.0	39.06
E_{st}/E_{1+2}	2.0	2.8		/	/
E_{st}/E_{1+2+9}	16.0	23.8		30.0	18.75
$E_{st}/ E_{1+2+9+12}$	/	/		10.0	6.25
E_{st}/E_8	6.0	15.4		25.0	22.88
E_{1+2+9}/E_{1+2+9}	4.0	2.89		/	2.25
E_8/E_8	2.0	1.21		5.0	3.06
E_8/E_{1+2}	2.0	0.44		/	/
E_8/E_{1+2+9}	10.0	3.74		/	5.25
Other		0.72			2.5
n	50	50		20	20
O_{st}/O_{st}	44.0	37.41		10.0	16.0
O_{st}/O_6	8.0	7.32		/	/
O_{st}/O_{22}	/	/		5.0	2.0
O_{st}/O_{3+4}	14.0	28.06		45.0	32.0
O_{st}/O_{3+4+1}	8.0	9.76		5.0	4.0

	4.0	2.44		/	/
O_{st}/O_{3+4+2}	4.0	2.44		/	/
O_{st}/O_{3+4+22}	/	/		5.0	10.0
O_6/O_{3+4}	2.0	2.76		/	/
O_6/O_{3+4+1}	2.0	0.96		/	/
O_{3+4}/O_{3+4}	14.0	5.29		10.0	16.0
O_{3+4}/O_{3+4+1}	2.0	3.68		/	4.0
O_{3+4}/O_{3+4+22}	/	/		15.0	10.0
O_{3+4+1}/O_{3+4+1}	2.0	0.64		/	0.25
O_{3+4+1}/O_{3+4+22}	/	/		5.0	1.25
Other		1.86			4.5
n	50	50		20	20

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383 Note: n = total number of karyotypes. "Other" stands for other karyotypic combinations

384 which were infrequent and were not found in the samples.

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386 **Table 2.** Frequencies (in percentage) of the observed (Obs.) and expected (Exp.)

387 karyotypes in both collections (1995 and 2010) from Petnica population.

	1995			2010	
	OBS.	EXP.		OBS.	EXP.
Karyotype	%	%		%	%
J_{st}/J_{st}	5.7	7.51		/	2.56
J_{st}/J_1	43.4	39.78		32.0	26.88
J_1/J_1	50.9	52.71		68.0	70.56
n	53	53		25	25

U_{st}/U_{st}	/	1.69		/	1.44
U_{st}/U_{1+2}	15.1	9.80		16.0	11.52
U_{st}/U_{1+2+6}	11.3	12.77		8.0	7.68
U_{st}/U_{1+8+2}	/	/		/	1.92
U_{1+2}/U_{1+2}	13.2	14.21		20.0	23.04
U_{1+2}/U_{1+2+6}	34.0	37.02		32.0	30.72
U_{1+2}/U_{1+8+2}	/	/		8.0	7.68
U_{1+2+6}/U_{1+2+6}	26.4	24.11		8.0	10.24
U_{1+2+6}/U_{1+8+2}	/	/		8.0	5.12
U_{1+8+2}/U_{1+8+2}	/	/		/	0.64
Other		0.4			/
n	53	53		25	25
E_{st}/E_{st}	17.0	16.48		4.0	9.0
E_{st}/E_{1+2}	3.8	3.09		12.0	3.6
E_{st}/E_{1+2+9}	30.1	31.42		24.0	20.4
$E_{st}/E_{1+2+9+12}$	/	/		8.0	3.6
E_{st}/E_8	13.2	13.80		8.0	14.4
E_{1+2}/E_{1+2+9}	1.9	2.94		/	4.08
E_{1+2}/E_8	1.9	1.29		/	2.88
E_{1+2+9}/E_{1+2+9}	17.0	14.98		8.0	11.56
E_8/E_8	3.8	2.89		4.0	5.76
E_8/E_{1+2+9}	11.3	13.16		28.0	16.32
$E_8/E_{1+2+9+12}$	/	/		4.0	2.88
Other		/			5.52

n	53	53		25	25
O_{st}/O_{st}	15.1	12.82		3.7	4.93
O_{st}/O_6	/	0.64		/	/
O_{st}/O_{22}	1.9	0.64		/	/
$O_{st}/O_{\underline{3+4}}$	30.1	28.35		29.7	18.91
$O_{st}/O_{\underline{3+4+1}}$	7.5	10.17		3.7	4.93
$O_{st}/O_{\underline{3+4+2}}$	1.9	2.0		3.7	0.84
$O_{st}/O_{\underline{3+4+8}}$	/	/		/	3.39
$O_{st}/O_{\underline{3+4+22}}$	/	4.08		/	6.57
$O_6/O_{\underline{3+4+1}}$	1.9	0.26		/	/
$O_{22}/O_{\underline{3+4}}$	/	0.71		/	/
$O_{\underline{3+4}}/O_{\underline{3+4}}$	18.8	15.68		7.4	18.18
$O_{\underline{3+4}}/O_{\underline{3+4+1}}$	3.8	11.25		3.7	9.46
$O_{\underline{3+4}}/O_{\underline{3+4+2}}$	1.9	2.22		/	1.62
$O_{\underline{3+4}}/O_{\underline{3+4+8}}$	/	/		14.8	6.3
$O_{\underline{3+4}}/O_{\underline{3+4+22}}$	5.7	4.51		22.2	12.61
$O_{\underline{3+4+1}}/O_{\underline{3+4+1}}$	5.7	2.02		3.7	1.23
$O_{\underline{3+4+1}}/O_{\underline{3+4+2}}$	1.9	0.8		/	0.42
$O_{\underline{3+4+1}}/O_{\underline{3+4+8}}$	/	/		/	1.64
$O_{\underline{3+4+1}}/O_{\underline{3+4+22}}$	1.9	1.62		7.4	3.29
$O_{\underline{3+4+2}}/O_{\underline{3+4+2}}$	/	0.08		/	/
$O_{\underline{3+4+22}}/O_{\underline{3+4+22}}$	1.9	0.32		/	2.19
Other		1.83			3.49
n	53	53		27	27

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389   Note: n = total number of karyotypes. õOtherö stands for other karyotypic combinations

390   which were infrequent and were not found in the samples.

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