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ORIGINAL ARTICLE

Evolutionary rate of adaptive chromosomal inversions in *Drosophila subobscura* (Diptera: Drosophilidae) in response to global warming

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Abstract. In the face of global warming, insects can employ various survival strategies. One possibility is that natural selection favors genetic combinations that are adaptive to this environmental situation. In some species, chromosomal inversions allow a particular combination of genes to be transmitted intact across generations, provided it is adaptive. *Drosophila subobscura* could therefore be considered a model species, given its rich chromosomal inversion polymorphism and its relationship with climate change. This polymorphism was studied in the Djerdap (Serbia) population in 2024, and its composition was found to have changed over time when the results were compared with those from 2001 and 2002. A significant decrease in 'cold' and an increase in 'warm' adapted inversions were observed when comparing 2001 and 2024. Also, the *CTI* (chromosomal thermal index) value for the whole karyotype in 2001 (0.162) was significantly different to that in 2024 (0.332) (adjusted $p = 0.0084$). Of the inversions studied, nine presented an observed frequency outside the interval expected assuming genetic drift. However, a change in agreement with global warming was detected in only five of these inversions, while the frequency of two 'non-thermal' adapted inversions also increased. Finally, an evolutionary rate was calculated by dividing the difference in frequency between 2024 and 2001 by the number of years that had elapsed. The values obtained for the 'cold', 'warm' and 'non-thermal' adapted were -0.0030126, 0.0030148 and -2.609×10^{-6}, respectively. These values are lower than those previously reported for the Apatin (Serbia) population.

INTRODUCTION

In terms of diversity, abundance, and biomass insects are the dominant form of animal life. A useful methodological approach to studying their adaptations to the changing environmental conditions of our planet is to collect time-series data from different geographic locations (Pélissié et al., 2018; Engelhardt et al., 2022; Pfenninger & Foucault, 2022; Ziesche et al., 2024; Cooke et al., 2025; Stroud & Ratcliff, 2025). One factor to which insects have to adapt is global warming (Williams, 2016; Garnas, 2018; Kellermann & van Heerwaarden, 2019; González-Tokman et al., 2020; Halsch et al., 2021; Eickermann et al., 2023; Harvey et al., 2023; deMayo & Ragland, 2025). A large number of studies on the thermal adaptation of insects, both in nature and under laboratory conditions, have been conducted using species of the *Drosophila* genus (see Fernández-Iriarte et al., 1999; Dahlgaard et al., 2001; Levitan, 2003; Levitan & Etges, 2005; Umina et al., 2005; Schaeffer, 2008; Soto et al., 2010; Hangartner et al., 2015; Kapun & Flatt, 2019; Singh, 2019; Nunez et al., 2024 and references therein). A useful genetic marker for studying thermal adaptation in this genus is chromosomal inversion polymorphism, introduced by Theodosius Dobzhansky (Dobzhansky, 1947, 1970; Lewontin et al., 1981), the father of experimental population genetics and a key contributor to the Synthetic Theory of Evolution. If chromosomal inversions are the genetic marker selected for studying thermal adaptation, an excellent model species is *D. subobscura* due to its rich inversion polymorphism, which can be observed throughout its karyotype with the exception of the dot chromosome (for instance, Menozzi & Krimbas, 1992; Orengo & Prevosti, 1996; Rodríguez-Trelles & Rodríguez, 1998; Castañeda et al., 2013; Balanyà et al., 2006, 2009; Rodríguez-Trelles et al., 2013; Zivanovic et al., 2023).

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In this species, 'warm', 'cold' and 'non-thermal' adapted inversions have been defined in Palearctic (Menozzi & Krimbas, 1992; Rego et al., 2010; Arenas et al., 2018) and American colonizing populations (Arenas et al., 2024).

In general, 'warm' adapted inversions increased in frequency, whereas 'cold' adapted inversions decreased in Palearctic populations from the central area of the species' distribution (Solé et al., 2002; Balanyà et al., 2004; Galludo et al., 2018; Rodriguez-Trelles & Tarrío, 2024), in Palearctic marginal populations (Khadem et al., 2022), on Madeira Island, an isolated island in the Atlantic Ocean (Madrenas et al., 2020) and in North and South American colonizing populations (Balanya et al., 2006, 2009; Rezende et al., 2010; Arenas et al., 2024). However, more information is still needed to properly understand how inversions adapt genetically to global warming. For instance, will *D. subobscura* populations located in different habitats or climatic conditions react in the same or similar way? Considering the species' karyotypes, will all chromosomes have the same adaptive potential, or will a particular inversion located on a certain chromosome show a predominant effect? In other words, are there major and minor inversions regarding thermal adaptation? Is there a threshold for the accumulation of 'warm' adapted inversions? To answer these questions, a large number of temporal series from populations located in the same region and also far away between them are needed to gather enough information.

For these reasons, the main purpose of this research was to collect again a large sample of *D. subobscura* flies from the Serbian population of Djerdap to obtain its chromosomal inversion polymorphism. This polymorphism was then compared at different levels (the whole karyotype and particular chromosomes) with those previously analyzed in the same month and place in 2001 and 2002 (Zivanovic, 2007). Furthermore, changes in the frequencies for 'warm', 'cold' and 'non-thermal' adapted inversions were studied and changes in several climatic variables (different temperatures, humidity and rainfall) were also analyzed, trying to assess the relation between both aspects, inversions and climate. Finally, evolutionary rates for chromosomal inversion polymorphism were computed and compared with values obtained from another Serbian population, Apatin (Zivanovic et al., 2019).

MATERIAL AND METHODS

Fly collection and analysis of inversions

D. subobscura flies were collected in the Djerdap gorge (Mali Kazan) of the Danube river (44°39′17″N and 22°18′29″E), located at 213 km from Belgrade. The trapping place was at an approximate altitude of 100 m a.s.l. The construction of a hydroelectric power plant on the border with Romania 53 years ago caused the creation of large lake at the entrance to the gorge. The large body of water produced an increase in air humidity. The Djerdap gorge was declared a national park in 1974. For this reason, it is protected from human influence, and flora and fauna are left undisturbed. The main vegetation consists of Balkan beech (*Fagus moesiaca*), the dominant tree, as well as hornbeam (*Carpinus orientalis*) and oak (*Querqus petraea*). Brushwood plants include *Hedera helix*, *Ilex aquifolium*, *Celtis australis*, *Ruscus aculeatus*, *Geranium macrorrhizum*, *Ceterach officinarum*, among others. According to the Köppen-Geiger climate classification (Kottek et al., 2006; Rubel & Kottek, 2011; Beck et al., 2018), the Djerdap region is classified as Cfb.

Sampling was carried out by netting flies over 40 traps filled with fermented apples by *Saccharomyces cerevisiae* from 3 p.m. to 8 p.m. *D. subobscura* individuals were collected from 31st May to 3rd June 2024 at exactly the same place where samples were obtained in 2001 and 2002. Once classified in the laboratory, wild males and sons of wild females were crossed in individual vials with virgin females from the Kussnacht reference strain. For all five chromosomes (A, E, J, U and O), this strain was homokaryotypic for the standard arrangements. All crosses were carried out under the following conditions: 18°C, 60% relative humidity and 12h/12h light/dark cycle. Chromosomal preparations were obtained from third instar larvae of the offspring, which were dissected and polytene chromosomes were stained and later squashed in an aceto-orcein solution. To correctly identify inversions, chromosomal reference maps were used (Kunze-Mühl & Müller, 1958; Krimbas, 1992, 1993). To attain the karyotypes with a probability greater than 0.99, at least eight larvae from the F_1 of each cross were analyzed. As is customary in *D. subobscura*, the term "inversion" refers to individual inversions or their combinations, known as arrangements. Finally, all chromosomal inversions were classified as 'C' (cold adapted), 'W' (warm adapted) and 'N' (non-thermal adapted) according to previously described criteria (Menozzi & Krimbas, 1992; Rego et al., 2010; Arenas et al., 2018).

Climatic data and statistical methodology

The climatic data from Djerdap were obtained from The Serbian Republic Hydrometeorological Service: mean temperature (Tmean), maximum temperature (Tmax), minimum temperature (Tmin), humidity and rainfall. For temperature, humidity and rainfall, the units used were centigrade degrees, percentage and millimeters of precipitation, respectively. In Djerdap, variations of climatic variables (Tmean, Tmax, Tmin, humidity and rainfall) over time (2001–2024) were studied with a temporal series.

Fisher's exact test was used (statistically significant $p < 0.05$) to compare the chromosomal inversion composition between different years, and analyze possible deviation in the observed frequencies of chromosomal karyotypes from those expected under Hardy-Weinberg conditions. To obtain the corresponding p values, a bootstrap procedure (100,000 runs) was used. The FDR correction (Benjamini & Hochberg, 1995) was applied whenever multiple comparisons were made (reported as significant for $p < 0.05$). Additionally, the index of free recombination (*IFR*) was calculated (Carson, 1955). The *basic* and *vegan* packages of R language (R Development Core Team, 2014) were used to carry out all statistical computations. Multivariate analyses were also computed with the Djerdap population and others from the Balkan region (Zivanovic et al., 2025 and references therein). The Mt. Parnes (Greece) (Araúz et al., 2009) and Font Groga (Barcelona, Spain) (Galludo et al., 2018) populations were used in the analyses as outgroups. Only inversions from the O chromosome, the most polymorphic of the species, were used. The procedure developed was to compute the Bhattacharyya (Bhattacharyya, 1946) distance between all populations and PCoA (Principal Coordinate Analysis) was carried out according to Balanyà et al. (2006) and Mestres et al. (2009). Additionally, a GEVA-Ward cluster was constructed using the same data, because it is considered a suitable method for inversion data (Irigoien et al., 2010; Zivanovic et al., 2016). Furthermore, the Pearson cophenetic correlation was computed to evaluate cluster accuracy while preserving the pairwise distances between the populations.

Finally, the chromosomal thermal adaptation of Djerdap population in different years (2001, 2002 and 2024) was measured by computing *CTI* (Chromosomal Thermal Index), which is defined by:

$$CTI = \frac{W - C}{W + C} = \frac{W - C}{TA}$$

where *W*, *C* and *TA* correspond to the total amount of 'warm', 'cold' and 'thermally adapted' chromosomes, respectively ($TA = W + C$). To test the hypothesis of equality in *CTI* indexes, a scored statistical test was also developed (complete information on this statistic and the associated test is presented in Arenas et al., 2018). Furthermore, the frequency variations over time of 'C' (cold adapted), 'W' (warm adapted) and 'N' (non-thermal adapted) were also analyzed.

Moreover, to ascertain whether the variations in the chromosomal inversions were produced by drift or by a deterministic factor (migration or selection) a computation was performed using the Wright-Fisher model (Crow & Kimura, 1970). According to the model, the expected mean frequency in generation *t*, $x^{(t)}$, given the initial mean frequency p_0 is,

$$x^{(t)} = p_0$$

and the variance can be estimated by

$$V_x^{(t)} = p_0(1 - p_0)[1 - (1 - \frac{1}{2N})^t],$$

which depends on the initial frequency of the inversion, the number of generations (*t*) and the effective population size (*N*). These parameters were estimated in natural populations of *D. subobscura* from Balkan and other central Palearctic areas. The average number of generations per year was estimated as five (Begon, 1976a; Mestres et al., 2001), and the time elapsed between both extreme samples from Djerdap was 23 years (2001–2024). Thus, the number of generations between both collections was estimated as 115. Additionally, a proper estimate of *N* would be 10,000 individuals in the peak of the species expansion (Begon, 1977; Begon et al., 1980; Mestres & Serra, 1991; Zivanovic et al., 2007; Araúz et al., 2009; Kurbalija-Novicic et al., 2013). For Djerdap population, this peak is reached for the months of May and June. Finally, the evolutionary rates for the inversion polymorphism were computed as the difference in frequency between the samples obtained in 2024 and 2001, and then divided by the number of years elapsed (Zivanovic et al., 2019).

RESULTS

Chromosomal inversion polymorphism and changes over time

The *D. subobscura* chromosomal inversion polymorphism obtained in Djerdap is presented in Table 1. Some inversions were not observed in previous studies carried out in this population (Zivanovic, 2007): $J_{\underline{3+4}}$, $O_{\underline{3+4}+\underline{6}}$ and $O_{\underline{3+4}+\underline{17}}$. The first one is considered to be related to arid climates (Krimbas, 1992, 1993), while the others were recently reported in Serbian populations: $O_{\underline{3+4}+\underline{6}}$ (Zivanovic et al., 2019, 2021, 2023, 2025) and $O_{\underline{3+4}+\underline{17}}$ (Zivanovic et al., 2021, 2023, 2025). The results of the comparisons between years (2001, 2002 and 2024) of chromosomal inversion polymorphism for each chromosome of the species' karyotype is presented in Table S1. For the whole karyotype, significant differences in inversion composition were observed between 2001 and 2002 compared to 2024. This effect was also observed for the U and O chromosomes and partially for the E (only the comparison between 2001 and 2024 was significant). The chromosomal karyotypes from Djerdap (Table 2) do not deviate from Hardy-Weinberg expectations: J chromosome $p = 0.9790$; U chromosome $p = 0.9165$; E chromosome $p = 0.9315$; O chromosome $p = 0.9690$; whole karyotype $p = 1$. Finally, the IFR value obtained in 2024 (82.32 ± 0.74) indicated that this population belongs to the central area of the *D. subobscura* distribution area. This value was very similar to those obtained in previous Djerdap studies (Zivanovic, 2007).

The results of multivariate analyses demonstrated that the Djerdap population belongs to the Serbian group of populations (Fig. 1). The three axes of the PCoA explain 36.78%, 18.65% and 10.91% of the variability, respectively. The four samples appear rather close in the graph, but the sample from 2024 is somewhat separated from the others (Fig. 1A). However, the cluster analysis results were not so conclusive (Fig. 1B), although the cophenetic correlation coefficient was good (0.762). The first partition of the tree separated the Font Groga (Barcelona) populations from the Balkan populations. The second partition separated the Djerdap 2024 (June) and 2001 (June) samples from the 2001 (August) and 2002 (June) samples. The third partition separated the June 2001 and June 2024 Djerdap samples.

Table 1. Chromosomal inversions frequencies of *Drosophila subobscura* from the Djerdap gorge (Serbia) population in June 2024.

Chrom. inversions	Thermal adapt.	n	%
A_{st}	C	71	47.3
A_1	C	48	32.0
A_2	W	31	20.7
Total		150	
J_{st}	C	53	17.7
J_1	W	242	80.7
$J_{\underline{3+4}}$	N	5	1.7
Total		300	
U_{st}	C	40	13.3
U_1	N	1	0.3
$U_{\underline{1}+\underline{2}}$	W	173	57.7
$U_{\underline{1}+\underline{2+3}}$	N	5	1.7
$U_{\underline{1+2+6}}$	N	75	25.0
$U_{\underline{1+8}+\underline{2}}$	W	6	2.0
Total		300	
E_{st}	C	84	28.0
E_8	N	89	29.7
$E_{\underline{1+2}}$	N	24	8.0
$E_{\underline{1+2+9}}$	W	84	28.0
$E_{\underline{1+2+9+12}}$	W	19	6.3
Total		300	
O_{st}	C	72	24.0
O_6	N	5	1.7
$O_{\underline{3+4}}$	W	142	47.3
$O_{\underline{3+4}+\underline{1}}$	W	21	7.0
$O_{\underline{3+4}+\underline{5}}$	N	3	1.0
$O_{\underline{3+4}+\underline{6}}$	N	14	4.7
$O_{\underline{3+4+8}}$	W	16	5.3
$O_{\underline{3+4}+\underline{17}}$	N	1	0.3
$O_{\underline{3+4}+\underline{22}}$	N	26	8.7
Total		300	

n – number of chromosomes, C – cold adapted, W – warm adapted, N – non-thermal adapted.

Table 2. Frequencies of chromosomal karyotypes of *D. subobscura* from the Djerdap gorge (Serbia) population in June 2024.

Karyotypes	*n*	%
J_{st}/J_{st}	6	4.0
J_{st}/J_{1}	41	27.3
J_{1}/J_{1}	98	65.3
$J_{1}/J_{\underline{3+4}}$	5	3.3
Total	150	
U_{st}/U_{st}	5	3.3
$U_{st}/U_{\underline{1}+\underline{2}}$	22	14.7
$U_{st}/U_{\underline{1}+\underline{2+3}}$	1	0.7
$U_{st}/U_{\underline{1+2+6}}$	7	4.7
U_{1}/U_{1+2}	1	0.7
$U_{\underline{1}+\underline{2}}/U_{\underline{1}+\underline{2}}$	54	36.0
$U_{\underline{1}+\underline{2}}/U_{\underline{1}+\underline{2+3}}$	3	2.0
$U_{\underline{1}+\underline{2}}/U_{\underline{1+2+6}}$	36	24.0
$U_{\underline{1}+\underline{2}}/U_{\underline{1+8}+\underline{2}}$	3	2.0
$U_{\underline{1+2+3}}/U_{\underline{1+2+6}}$	1	0.7
$U_{\underline{1+2+6}}/U_{\underline{1+2+6}}$	14	9.3
$U_{\underline{1+2+6}}/U_{\underline{1+8}+\underline{2}}$	3	2.0
Total	150	
E_{st}/E_{st}	11	7.3
$E_{st}/E_{\underline{1+2}}$	8	5.3
$E_{st}/E_{\underline{1+2+9}}$	23	15.3
$E_{st}/E_{\underline{1+2+9+12}}$	7	4.7
E_{st}/E_{8}	24	16.0
$E_{\underline{1+2}}/E_{\underline{1+2}}$	2	1.3
$E_{\underline{1+2}}/E_{\underline{1+2+9}}$	3	2.0
$E_{\underline{1+2}}/E_{8}$	9	6.0
$E_{\underline{1+2+9}}/E_{\underline{1+2+9}}$	15	10.0
$E_{\underline{1+2+9}}/E_{\underline{1+2+9+12}}$	1	0.7
$E_{\underline{1+2+9}}/E_{8}$	27	18.0
$E_{\underline{1+2+9+12}}/E_{\underline{1+2+9+12}}$	3	2.0
$E_{\underline{1+2+9+12}}/E_{8}$	5	3.3
E_{8}/E_{8}	12	8.0
Total	150	
O_{st}/O_{st}	12	8.0
O_{st}/O_{6}	1	0.7
$O_{st}/O_{\underline{3+4}}$	39	26.0
$O_{st}/O_{\underline{3+4}+\underline{1}}$	1	0.7
$O_{st}/O_{\underline{3+4}+\underline{6}}$	3	2.0
$O_{st}/O_{\underline{3+4}+\underline{22}}$	4	2.7
$O_{6}/O_{\underline{3+4}}$	3	2.0
$O_{6}/O_{\underline{3+4}+\underline{5}}$	1	0.7
$O_{\underline{3+4}}/O_{\underline{3+4}}$	31	20.7
$O_{\underline{3+4}}/O_{\underline{3+4}+\underline{1}}$	9	6.0
$O_{\underline{3+4}}/O_{\underline{3+4}+\underline{5}}$	1	0.7
$O_{\underline{3+4}}/O_{\underline{3+4}+\underline{6}}$	5	3.3
$O_{\underline{3+4}}/O_{\underline{3+4+8}}$	10	6.7
$O_{\underline{3+4}}/O_{\underline{3+4}+\underline{17}}$	1	0.7
$O_{\underline{3+4}}/O_{\underline{3+4}+\underline{22}}$	12	8.0
$O_{\underline{3+4}+\underline{1}}/O_{\underline{3+4}+\underline{1}}$	3	2.0
$O_{\underline{3+4}+\underline{1}}/O_{\underline{3+4+8}}$	1	0.7
$O_{\underline{3+4}+\underline{1}}/O_{\underline{3+4}+\underline{22}}$	4	2.7
$O_{\underline{3+4}+\underline{5}}/O_{\underline{3+4}+\underline{22}}$	1	0.7
$O_{\underline{3+4}+\underline{6}}/O_{\underline{3+4}+\underline{6}}$	2	1.3
$O_{\underline{3+4}+\underline{6}}/O_{\underline{3+4}+\underline{22}}$	2	1.3
$O_{\underline{3+4+8}}/O_{\underline{3+4+8}}$	1	0.7
$O_{\underline{3+4+8}}/O_{\underline{3+4}+\underline{22}}$	3	2.0
Total	150	

n – number of karyotypes.

Climatic variables and chromosomal inversion changes

It was observed that all temperatures increased over time: Tmean (*p* = 0.095, with a slope of 0.072), Tmax (*p* = 0.114, with a slope of 0.089) and Tmin (*p* = 0.027, with a slope of 0.069). The latter was the only significant temperature. The difference between Tmax and Tmin remained rather constant throughout the years studied (*p* = 0.670, with a slope of 0.021). Finally, humidity exhibited an erratic pattern with an increasing trend (*p* = 0.163, with a slope of 0.240). Rainfall was erratic without a discernible trend (*p* = 0.767, with a slope of −0.502). Graphs showing the fluctuation of climatic variables over time (2001–2024) are included in the supplementary material (Fig. S1).

Table 3. Statistical comparisons between the *CTI* values computed for the June Djerdap population (2001, 2002 and 2024). In the rows, the values of the statistical test are presented and the corresponding adjusted *p* values are shown below in brackets. Significant values are in bold.

	2001	2002	2024
2001	–	0.0798 (0.1197)	**0.0028** (**0.0084)**
2002		–	0.6017 (0.6017)
2024			–

For the thermally adapted inversions, the *CTI* values were 0.162, 0.343, 0.299 and 0.332 for Djerdap 2001 (June), 2001 (August), 2002 (June) and 2024 (June), respectively. The highest value corresponded to August 2001. However, when comparing equivalent samples in June, an increase was observed. This increase was significant in the comparison between 2001 and 2024 (Table 3). Furthermore, it is possible to compare the composition of thermal adapted inversions (‘cold’, ‘warm’ and ‘non-thermal’) between these three populations. Significant results were obtained in the comparisons between 2001 and 2024 for all groups of thermal adapted inversions (Table S2). Additionally, changes in the frequency of individual chromosomal inversions over time (2001–2024) were examined to determine if they were caused by genetic drift or a directional evolutionary factor, such as migration or natural selection (Table S3). According to global warming expectations, variations in frequency were observed in 11 out of 15 cases (4 for ‘cold’ and 7 for ‘warm’), with a decrease of ‘cold’ and an increase

Table 4. The evolutionary rates of frequency change for the ‘cold’, ‘warm’ and ‘non-thermal’ inversions of *D. subobscura* from the Djerdap population (2001–2024). In the last column, the rate of change is presented.

Chromosomes	2001	2024	*Rate*
A Cold	0.90385	0.79333	−0.00481
A Warm	0.09615	0.20667	0.00481
J Cold	0.24038	0.17667	–0.00277
J Warm	0.75962	0.80667	0.00205
J Non-thermal	0.0	0.01667	0.00072
U Cold	0.14423	0.13333	–0.00047
U Warm	0.43269	0.59667	0.00713
U Non-thermal	0.42308	0.27000	−0.00666
E Cold	0.46153	0.28000	–0.00789
E Warm	0.26923	0.34333	0.00322
E Non-thermal	0.26923	0.37667	0.00467
O Cold	0.24038	0.24000	–0.00002
O Warm	0.62500	0.59667	–0.00123
O Non-thermal	0.13462	0.16333	0.00125

Rate has been computed as the difference in frequency between the samples obtained in 2024 and 2001, and then divided by the number of years elapsed (in this case 23).

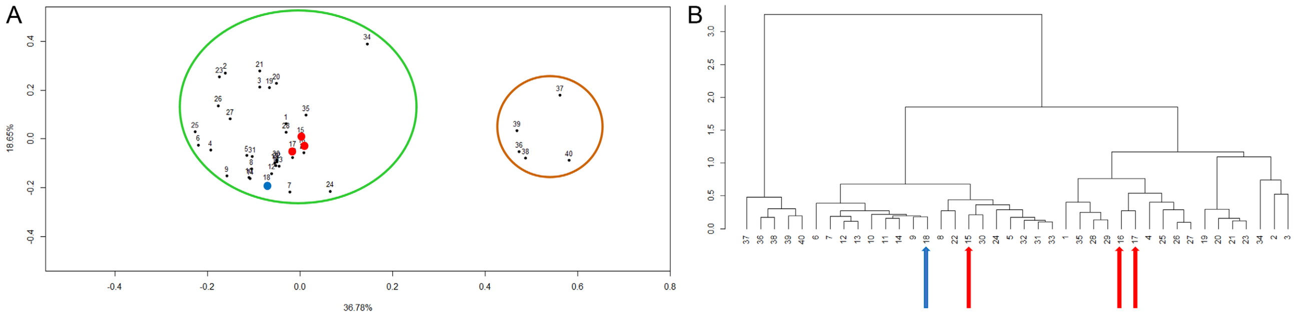

Fig. 1. Results of multivariate analyses. (A) Principal Coordinate Analysis of the Balkan and Font Groga (Barcelona) populations using the O chromosomal inversions. Sample numbers: 1 – Mt. Parnes 2009 (Greece); 2 – Apatin 1994; 3 – Apatin 2008; 4 – Apatin 2009; 5 – Apatin 2018; 6 – Avala 2003; 7 – Avala 2004 (June); 8 – Avala 2004 (Sept.); 9 – Avala 2005; 10 – Avala 2011; 11 – Avala 2014; 12 – Avala 2015; 13 – Avala 2016; 14 – Avala 2017; 15 – Djerdap 2001 (June); 16 – Djerdap (August); 17 – Djerdap (2002); 18 – Djerdap (2024); 19 – Jastrebac Mt. (beech) 1990; 20 – Jastrebac Mt. (beech) 1993); 21 – Jastrebac Mt. (beech) 1994; 22 – Jastrebac Mt. (beech) 2023; 23 – Jastrebac Mt. (oak) 1990; 24 – Jastrebac Mt. (oak) 2023; 25 – Kamariste 1996; 26 – Petnica (May) 1995; 27 – Petnica (June) 1995; 28 – Petnica (August) 1995; 29 – Petnica 2010; 30 – Petnica 2019; 31 – Petnica 2020; 32 – Petnica 2021; 33 – Petnica 2022; 34 – Fruska Gora; 35 – Zanjic (Montenegro) 1997; 36 – Font Groga 2011; 37 – Font Groga 2012; 38 – Font Groga 2013; 39 – Font Groga 2014; 40 – Font Groga 2015. The red color dots indicate Djerdap from 2001 and 2002, whereas the blue color dot indicates Djerdap 2024. The green circle contains all Balkan populations, and the brown circle contains all Font Groga (Barcelona) populations. (B) Phylogenic tree using the O chromosomal inversions from Djerdap and other Balkan and Font Groga (Barcelona) populations. The red color arrows indicate Djerdap from 2001 and 2002, and the blue color arrow indicates Djerdap 2024.

of ‘warm’ adapted inversions. However, of all inversions studied, only nine presented a frequency p_{obs} (the observed frequency in 2024) outside the interval defined by p_{exp} multiplied by two times the standard deviation generated by the model. Of the nine inversions with p_{obs} outside the defined interval, two were ‘cold’ adapted, with p_{obs} below the lower limit of the interval. Five belonged to the ‘warm’ group, with three presenting p_{obs} above the higher limit and two below. Two were ‘non-thermal’ adapted, one above the higher limit and the other below.

Evolutionary rates of thermal adapted inversions

Estimates of evolutionary rates for ‘cold’, ‘warm’ and ‘non-thermal’ adapted inversions for each chromosome of the *D. subobscura* karyotype are presented in Table 4. Global estimates of evolutionary rates were also computed for the whole karyotype: ‘cold’ adapted inversions, frequency 2024 = 0.27259, frequency 2001 = 0.34188, evolutionary rate = –0.0030126; ‘warm’ adapted inversions, frequency 2024 = 0.54370, frequency 2001 = 0.47436, evolutionary rate = 0.0030148; ‘non-thermal’ adapted inversions, frequency 2024 = 0.18370, frequency 2001 = 0.18376, evolutionary rate = -2.609×10^{-6}.

DISCUSSION

Unfortunately, global warming is a fact. In the present study of the Djerdap location, an increase in all temperatures analyzed was reported, and the increment for Tmin was significant. This result was also detected in other Serbian populations (for a revision see, Zivanovic et al., 2023, 2025 and references therein). *D. subobscura* can currently survive and reproduce in the context of global warming, and the gene content of its chromosomal inversions is likely a key factor in its adaptation. In the case of Djerdap, the composition of its chromosomal inversion polymorphism is typical of Serbian *D. subobscura* populations, as demonstrated by the PCoA and cluster analyses (Fig. 1).

In the 2024 sample, the presence of the $J_{\underline{3+4}}$ inversion for the first time indicated a trend toward more arid conditions (Krimbas 1992, 1993). This inversion has also recently been reported in other Serbian populations (Zivanovic et al., 2021, 2023, 2025). This fact would indicate that the $J_{\underline{3+4}}$ inversion reached Djerdap from other populations (by migration or gene flow) and is likely adaptive to the current climatic conditions. There is a substantial evidence regarding dispersal, migration and gene flow capacities of *D. subobscura* (Greuter, 1963; Begon, 1976b; Loukas & Krimbas, 1979; Serra et al., 1987; Ayala et al., 1989; Prevosti et al., 1989; Latorre et al., 1992; Krimbas, 1993; Pascual et al., 2001; Zivanovic et al., 2007; Pegueroles et al., 2013). In insects, one strategy for overcoming the effects of global warming is to move to other places (for instance, Ubukata, 2000; Menéndez, 2007; Guo et al., 2011; Zeng et al., 2020; Juhász et al., 2023). Another possibility is genetic adaptation to global warming through natural selection. Over time, genes that confer adaptation to a warming scenario would increase in frequency, and if they are anchored in inversions, the frequency of the ‘warm’ adapted inversions would increase accordingly. As mentioned in the Introduction, it is well known that the *D. subobscura* chromosomal inversion polymorphism changed over time in response to climate conditions. In the Djerdap population, changes over time in chromosomal composition were observed at both the overall level (Table S1) and when considering only thermally adapted inversions (Table S2). Analyzing the first aspect, significant changes in inversion composition were obtained for the whole karyotype when comparing data from 2024 with 2001 or 2002. However, inversion composition was significant for chromosmes U and O chromosomes in the same comparisons (2024 with regard 2001 and 2002), but only for the E chromosome in the 2024 versus 2001 comparison. No comparison was found to be significant for the A and J chromosomes.

When focusing on thermal adapted inversions, all comparisons between 2024 and 2001 were significant, even for “non-thermal” adapted inversions. Another aspect studied was the *CTI*, and the values obtained for Djerdap (June of 2001, 2002 and 2024) were in the middle range of those obtained in Serbian populations belonging to the Cfb climate group (Zivanovic et al., 2023). However, significant differences were detected between 2024 and 2001 Djerdap samples (Table 4). Thus, solid evidence was obtained in this population demonstrating that the chromosomal inversion composition has changed in accordance with expectations of global warming. Similar patterns of variation were observed in other *D. subobscura* populations (Orengo & Prevosti, 1996; Rodríguez-Trelles & Rodríguez, 1998; Solé et al., 2002; Balanyà et al., 2004, 2006, 2009; Rodríguez-Trelles et al., 2013; Zivanovic et al., 2015; 2021, 2023; Galludo et al., 2018; Madrenas et al., 2020; Khadem et al., 2022; Arenas et al., 2024; Rodriguez-Trelles & Tarrío, 2024). In the Djerdap population, it was interesting to ascertain the role of particular inversions in *D. subobscura* thermal adaptation. Those inversions responding to directional evolutionary factors, such as migration or selection, appeared significant in Table S3. Only two ‘cold’ adapted inversions appeared significant (A_1 and E_{st}) and both have changed as expected due to global warming (decreasing). Three of the five ‘warm’ adapted inversions agreed with the expectations (A_2, $U_{\underline{1+2}}$ and $E_{\underline{1+2+9+12}}$), while two decreased ($U_{\underline{1+8+2}}$ and $O_{\underline{3+4+1}}$). Perhaps, it was a sampling effect due to the low frequency of both inversions in the population. Finally, a couple of ‘non-thermal’ adapted inversions presented significant differences according to the model ($U_{\underline{1+2+6}}$ and E_8). It is well known that both irradiated in all directions from a central area where their frequency is maximum. For $U_{\underline{1+2+6}}$, the maximum frequency is found in Greece, and for E_8, is in Anatolia (Krimbas, 1992, 1993). When these results were compared with those from Apatin for the period 1994–2018 (Zivanovic et al., 2019), the significant inversions in both studies were A_2, $U_{\underline{1+2}}$, $U_{\underline{1+2+6}}$, E_{st} and E_8. Interestingly, there is no match for the chromosomes J and O, even though they exhibit thermal adaptation. Further research is needed to generate replicates that would improve our understanding of the behavior of thermal adapted inversions. Moreover, it should be noted that thermal adaptation appears to have an accumulative effect of thermal adapted inversions. While different kind of interactions, such as epistasis and linkage disequilibrium, cannot be ruled out, adaptation based on inversions would act mainly as an additive process (Zivanovic et al., 2014, 2016). Therefore, the total number of ‘warm’ inversions is a relevant factor in adaptation. However, this accumulation could not be ad infinitum, as fitness genes could be in linkage disequilibrium with ‘cold’ adapted inversions. For this reason, these inversions must remain at a certain frequency in *D. subobscura* populations (Zivanovic et al., 2025).

Another interesting aspect is the rhythm of accumulation of ‘warm’ adapted inversions, thus increasing their frequency and generating the adaptation to global warming scenario. In Djerdap, the evolutionary rate for ‘cold’ and ‘warm’ adapted chromosomes is almost equal, but have different signs; thus, the first group decreased at the same rate as the second increased. The ‘non-thermal’ adapted inversion presented a negligible rate of change. These results generally align with the global warming scenario. When the rates were studied for individual chromosomes (Table 4), a similar effect was observed for the A, J and E. The magnitude of the rate is equivalent to the decrease of ‘cold’ and increase of ‘warm’ thermal adapted inversions. However, this was not the case for the U and O chromosomes. For the U chromosome, the rate of increase for ‘warm’ adapted in one order of magnitude greater than for ‘cold’ adapted. The situation for the O chromosome is particular, because the rate of change for the ‘cold’ adapted inversions was negligible, the ‘warm’ decreased and the ‘non-thermal’ increased, and in the same rate as the ‘warm’ inversions. When these results are compared with the only equivalent available study, the Serbian population of Apatin for a period of 24 years (1994–2018) (Zivanovic et al., 2019), the order of magnitude of the different rates is slightly higher than those obtained in Djerdap. The Apatin study reported analogous results to those observed in Djerdap for the A chromosome. However, the inversions of the J, U and E chromosomes changed as expected, but at a lower rate than in Apatin. Finally, the results for the O chromosome in Apatin align with global warming expectations. Several considerations must be taken into account, for instance, the computation assumes that the rate remains constant over time. This could be an oversimplification of what happens in actual natural populations. Also, the lower rates in Djerdap could be a consequence of a possible threshold in the accumulation of ‘warm’ and loss of ‘cold’ adapted inversions (Zivanovic et al., 2025). For these reasons, it would be needed to compute these evolutionary rates in other *D. subobscura* populations to confirm the existing findings. These future populations should also be studied over a long temporal series, including an analysis of inversions polymorphism over shorter time periods, to ascertain whether the rate is constant or variable. It would also be useful to estimate these rates in other *Drosophila* species in which inversions have been described that are, or maybe could be, related to thermal adaptation (for instance, Brncic & Napp, 1980; Fernández-Iriarte et al., 1999; Dahlgaard et al., 2001; Levitan, 2003; Ananina et al., 2004; Umina et al., 2005; Levitan & Etges, 2005; Schaeffer, 2008; Soto et al., 2010; Batista et al., 2012; Hangartner et al., 2015; Salceda et al., 2015; Reis et al., 2018; Kapun & Flatt, 2019; Esteve et al., 2020; Singh & Singh, 2021; Poikela et al., 2024). To generate comparable results in future analyses, the rate could be calculated by dividing the difference in frequencies by the number of generations instead of the number of years. This simple computation could be carried out in other insect species with chromosomal inversions, some of which seem to be related with thermal adaptation (for instance see, Ayala et al., 2014; Stegniy et al., 2016; Mérot et al., 2021; McCulloch & Waters, 2023; Sharakhov & Sharakhova, 2024; Ma et al., 2024; Pita et al., 2025, Couper et al., 2025).

In summary, the inversion chromosomal polymorphism of Djerdap has changed over time (2001–2024). Considering the whole karyotype, 'cold' and 'warm' adapted inversions have changed their frequencies in agreement with the global warming scenario. However, the frequencies of particular chromosomes and inversions showed unexpected variations. It should be noted that it is the entire set of karyotype inversions that most likely generates thermal adaptation. The evolutionary rate estimated for the inversion polymorphism in Djerdap is lower than that previously reported in Apatin (Serbia). Further studies incorporating the aforementioned improvements are necessary in order to better understand how *D. subobscura* adapts to climate change.

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Table S1. Comparisons between years (2001, 2002 and 2024) of the chromosomal inversion polymorphism from Djerdap for each chromosome of the species (A, J, U, E and O) and for the whole karyotype. The corresponding *p* and the adjusted *p* values are presented. Significant values appear in bold.

	p	*Adjusted p*
A chromosome		
2001 vs. 2002	0.7251	0.7251
2001 vs. 2024	0.0780	0.2339
2002 vs. 2024	0.3463	0.5195
J chromosome		
2001 vs. 2002	0.7046	0.7046
2001 vs. 2024	0.1964	0.5892
2002 vs. 2024	0.5837	0.7046
U chromosome		
2001 vs. 2002	0.3863	0.3863
2001 vs. 2024	**0.0005**	**0.0015**
2002 vs. 2024	**0.0115**	**0.0172**
E chromosome		
2001 vs. 2002	0.1674	0.2511
2001 vs. 2024	**0.0035**	**0.0105**
2002 vs. 2024	0.2634	0.2634
O chromosome		
2001 vs. 2002	0.5282	0.5282
2001 vs. 2024	**0.0125**	**0.0262**
2002 vs. 2024	**0.0175**	**0.0262**
Whole karyotype		
2001 vs. 2002	0.3448	0.3448
2001 vs. 2024	**0.0005**	**0.0015**
2002 vs. 2024	**0.0035**	**0.0052**

Table S2. For Djerdap populations, comparisons between samples of June (2001, 2002 and 2024) for all chromosomes (A, J, U, E and O), considering the classification of thermal adapted inversions (‘cold’, ‘warm’ and ‘non-thermal’ adapted). The values of *p* and adjusted *p* are shown and significant values appear in bold.

COLD INV.	*p*	*Adjusted p*
2001 vs. 2002	*0.1639*	*0.2439*
2001 vs. 2024	**0.0030**	**0.0090**
2002 vs. 2024	0.2439	0.2439
WARM INV.		
2001 vs. 2002	0.1669	0.2279
2001 vs. 2024	**0.0030**	**0.0090**
2002 vs. 2024	0.2279	0.2279
NON-THERMAL INV.		
2001 vs. 2002	0.1714	0.2544
2001 vs. 2024	**0.0060**	**0.0180**
2002 vs. 2024	0.2544	0.2544

Table S3. The Fisher-Wright expectations for the *D. subobscura* inversion chromosomal frequencies from Djerdap (2001–2024).

		p_0	p_{exp}	2*SD	p_{exp} ± 2*SD	p_{obs}
A_{st}	C	0.42	0.42	0.07	[0.348, 0.498]	0.47
A_1	C	0.48	0.48	0.08	[0.404, 0.556]	**0.32**
A_2	W	0.10	0.10	0.04	[0.052, 0.142]	**0.21**
J_{st}	C	0.24	0.24	0.06	[0.175, 0.305]	0.18
J_1	W	0.76	0.76	0.06	[0.695, 0.825]	0.81
$J_{\underline{3+4}}$	N	0.00	0.00	N/A	N/A	0.02
U_{st}	C	0.14	0.14	0.05	[0.091, 0.197]	0.13
U_1	N	0.00	0.00	N/A	N/A	0.00
$U_{\underline{1}+\underline{2}}$	W	0.34	0.34	0.07	[0.265, 0.408]	**0.58**
$U_{\underline{1}+\underline{2+3}}$	N	0.01	0.01	0.01	[–0.005, 0.023]	0.02
$U_{\underline{1+2+6}}$	N	0.41	0.41	0.07	[0.339, 0.489]	**0.25**
$U_{\underline{1+8}+\underline{2}}$	W	0.10	0.10	0.04	[0.051, 0.141]	**0.02**
E_{st}	C	0.46	0.46	0.08	[0.386, 0.538]	**0.28**
E_8	N	0.21	0.21	0.06	[0.150, 0.274]	**0.30**
$E_{\underline{1+2}}$	N	0.06	0.06	0.04	[0.023, 0.093]	0.08
$E_{\underline{1+2+9}}$	W	0.26	0.26	0.07	[0.193, 0.325]	0.28
$E_{\underline{1+2+9+12}}$	W	0.01	0.01	0.01	[–0.005, 0.023]	**0.06**
O_{st}	C	0.24	0.24	0.06	[0.175, 0.305]	0.24
O_6	N	0.02	0.02	0.02	[–0.002, 0.040]	0.02
O_7	N	0.01	0.01	0.02	[–0.005, 0.025]	0.00
$O_{\underline{3+4}}$	W	0.45	0.45	0.08	[0.377, 0.527]	0.47
$O_{\underline{3+4}+\underline{1}}$	W	0.14	0.14	0.05	[0.091, 0.197]	**0.07**
$O_{\underline{3+4}+\underline{2}}$	N	0.02	0.02	0.02	[–0.002, 0.040]	0.00
$O_{\underline{3+4}+\underline{5}}$	N	0.00	0.00	N/A	N/A	0.01
$O_{\underline{3+4}+\underline{6}}$	N	0.00	0.00	N/A	N/A	0.05
$O_{\underline{3+4}+\underline{7}}$	N	0.01	0.01	0.02	[–0.005, 0.025]	0.00
$O_{\underline{3+4+8}}$	W	0.03	0.03	0.03	[0.004, 0.054]	0.05
$O_{\underline{3+4}+\underline{17}}$	N	0.00	0.00	N/A	N/A	0.00
$O_{\underline{3+4+22}}$	N	0.08	0.08	0.04	[0.037, 0.117]	0.09

The variables are: p_0 (initial frequencies observed in 2001), p_{exp} (expected frequencies in 2024 according to the model) and p_{obs} (observed frequencies in 2024). SD stands for standard deviation calculated according to the model. If values of p_{obs} are not included in the interval p_{exp} ± 2*SD, then are shown in bold. C – cold adapted, W – warm adapted, N – non-thermal adapted, N/A – not applicable.

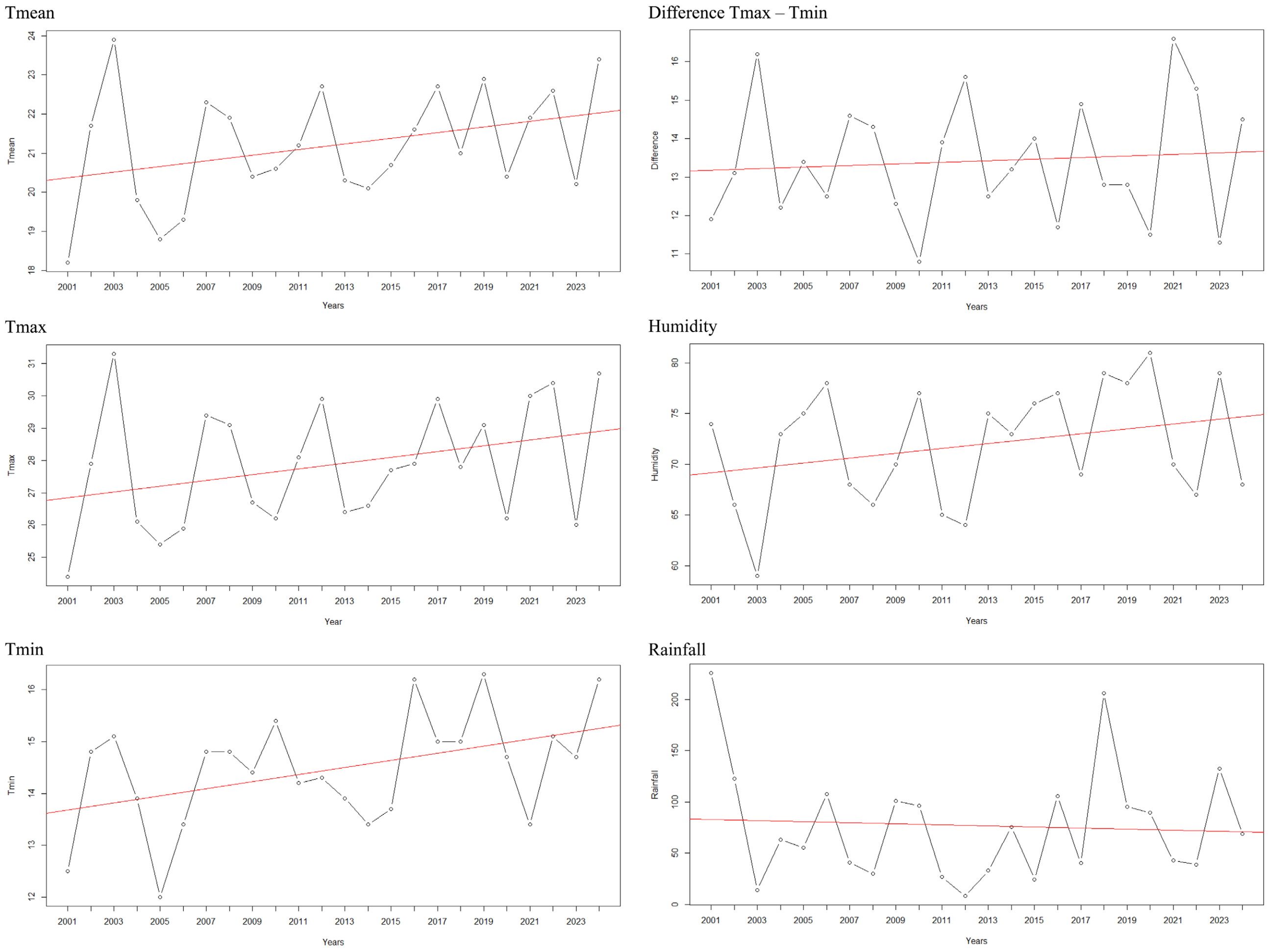

Fig. S1. Fluctuations of climatic variables over time (2001–2024): Tmean, Tmax, Tmin, Difference Tmax – Tmin, Humidity and Rainfall.