Associations between environmental variability and inversion polymorphism of *Drosophila subobscura*: meta-analysis of populations from the Central Balkans

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ABSTRACT: Ecological and evolutionary factors, together with abiotic conditions, affect biogeographic patterns of genetic entities. The spatial and temporal variability of chromosomal inversions of *Drosophila subobscura* suggests that this species can serve as a good model for studying the effects of environmental change on the genetic structure of natural populations. A comprehensive meta-analysis of the association of environmental and climatic variables with inversion diversity patterns was performed on 20 *D. subobscura* populations from the central part of the Balkan Peninsula. Environmental data consisted of 3 sets of variables related to temperature and precipitation, extracted from 2 climatic databases, averaged over a 3 month period, and using biological instead of calendar dates of sampling. Arrangement frequency patterns are likely driven by a synergistic effect of factors related to temperature and precipitation. The frequencies of standard chromosomal arrangements tend to co-vary positively with precipitation, whereas parameters related to temperature appear to favor higher frequencies of the inverted and more complex chromosomal arrangements. A complex relationship among local environmental variables is evident from the results and reflects the probable effect of an altitudinal shift; the altitudinal gradient of inversions is different from their latitudinal gradient. The pattern of inversions is generally not associated with environmental variables, and a particular inversion cannot be a predictive genetic marker of global climate change. Populations in different habitats are subjected to habitat-specific selection regimes, while demographic factors and population history also affect the genetic variability pattern observed.

KEY WORDS: Adaptation · Climate · Chromosomal polymorphism · Thermal response

1. INTRODUCTION

Insects play a fundamental role in most ecosystems, and environmental changes at global and local scales affect insect species and populations, with resulting impacts on ecosystems. Ecological and evolutionary factors, together with abiotic and biotic environmental conditions, shape the spatial and temporal changes in genetic structures of populations. Genetic markers, such as polymorphisms at chromosomal, nuclear DNA, and mtDNA levels, provide information on the patterns of changes in genetic variability of populations, and possibly predict future outcomes.

Chromosomal inversion polymorphisms have been detected in a number of insects (Hoffmann et al. 2004), and data suggest that they can serve as indica-

tors of evolutionary dynamics in natural populations, and play an important role in speciation, as in the *Anopheles gambiae* complex (Coluzzi et al. 2002). A combination of alleles within an inversion is 'protected' from meiotic recombination as a coadaptive complex, and has been shown to be favored under selection (Kirkpatrick & Barton 2006). Inversion polymorphism has been studied in many *Drosophila* species (Dobzhansky 1971, Lewontin et al. 1981, Fontdevila et al. 1982, Krimbas & Powell 1992, Hasson et al. 1995). Selection has a role in shaping inversion frequencies in *Drosophila* clines in association with climatic gradients, as documented by Dobzhansky (1971) on the spatial and temporal distribution pattern of arrangements on the third chromosome of *D. pseudoobscura*. The genetic variation maintained by spatially variable selection is confirmed in *D. melanogaster* and *D. buzatti*, with parallel patterns of the latitudinal clines of inversion polymorphisms observed on different continents (Knibb 1982, Ruiz et al. 1986, Anderson et al. 2005, Hoffmann & Weeks 2007). Genomic-scale and whole-genome sequence studies (Turner et al. 2008, Fabian et al. 2012, Kapun et al. 2016) provide strong evidence that spatially and temporally varying selection maintains inversion frequency clines in *D. melanogaster*.

D. subobscura has a broad Palearctic distribution, and in the late 1970s it colonized South and North America, where it spread rapidly and successfully. Population genetic structure screening studies suggest that different genetic markers show different amounts of geographic variation, i.e. allozymes (Marinkovic et al. 1978, Castro et al. 1999), mtDNA (Latorre et al. 1986, Afonso et al. 1990, García-Martínez et al. 1998, Castro et al. 1999), and nuclear DNA polymorphisms (Pascual et al. 2000, 2001, Kurbalija Novicic et al. 2011). Exceptionally numerous inversions in *D. subobscura* are simple and complex gene arrangements on all 4 autosomes and sex chromosomes, and are the most informative markers of the adaptive change among *Drosophila* species (Prevosti et al. 1988, Krimbas 1993). The available results on spatial and temporal variability in *D. subobscura* inversion frequencies suggest that this is a good model species for studying the effects of environmental change on the genetic structure of natural populations (Balanyá et al. 2006, Stamenkovic-Radak et al. 2008, Rezende et al. 2010).

The focus of the present paper is the association of climatic variables with inversion diversity patterns of *D. subobscura* populations from the central part of the Balkan Peninsula. Selection by climatic variables seems to be the leading factor that shaped the overall pattern of geographic frequency distribution of inversions in *D. subobscura* (Menozzi & Krimbas 1992). It remains uncertain to what degree ecological and evolutionary factors, together with abiotic varying conditions, affect biogeographic patterns of genetic entities. The present paper provides a meta-analysis of the association of environmental and climatic variables, such as latitude, longitude, altitude, temperature, precipitation, and humidity, with chromosomal inversion frequency in 20 *D. subobscura* populations from the Central Balkans.

2. MATERIALS AND METHODS

2.1. Population and environmental data

Populations of *Drosophila subobscura* used for the meta-analysis were sampled from 20 localities, ranging from 45.67° to 42.40° N latitude and from 22.62° to 18.58° E longitude (Fig. 1, and see Table S1a in Supplement 1 at www.int-res.com/articles/suppl/c077p205_supp.pdf). The sampling locations differ in altitude and ecological and vegetation characteristics of the habitat. Inversion polymorphism data are either from previously published studies (Zivanovic et al. 2002, Kalajdžić et al. 2006, Zivanovic 2007, Kenig et al. 2010, Kurbalija Novicic et al 2013) or remain unpublished up to the publication of this study (Table S1a). Regardless of the data origin, experimental procedures, including field sampling, laboratory rearing of flies, chromosomal preparations, and inversion determination, were done in the same laboratory, following the same protocols. Flies were sampled mostly in the second half of June, and dates are taken from laboratory and field records and are included in Table S1a. Flies were attracted by 10–12 fermented mashed fruit traps (80% apple) on plates distributed over an area of about 400 m^2, and swiped with a net during the late afternoon/evening peak activity, approximately 18:00–20:00 h.

Environmental data used in this study consisted of 3 separate data sets. The first and second datasets were climatic variables related to temperature and precipitation extracted from the WorldClim database from the European square 16 raster grids; resolution is 30 arc-seconds, which is approximately 1 km (www.worldclim.org/; for details on WorldClim database usage methods see Hijmans et al. 2005, Hijmans & van Etten 2012, Fick & Hijmans 2017). WorldClim data represent monthly averages across 30 yr of observation. These data enable evaluation of the impact of long-term climatic trends for particular locations,
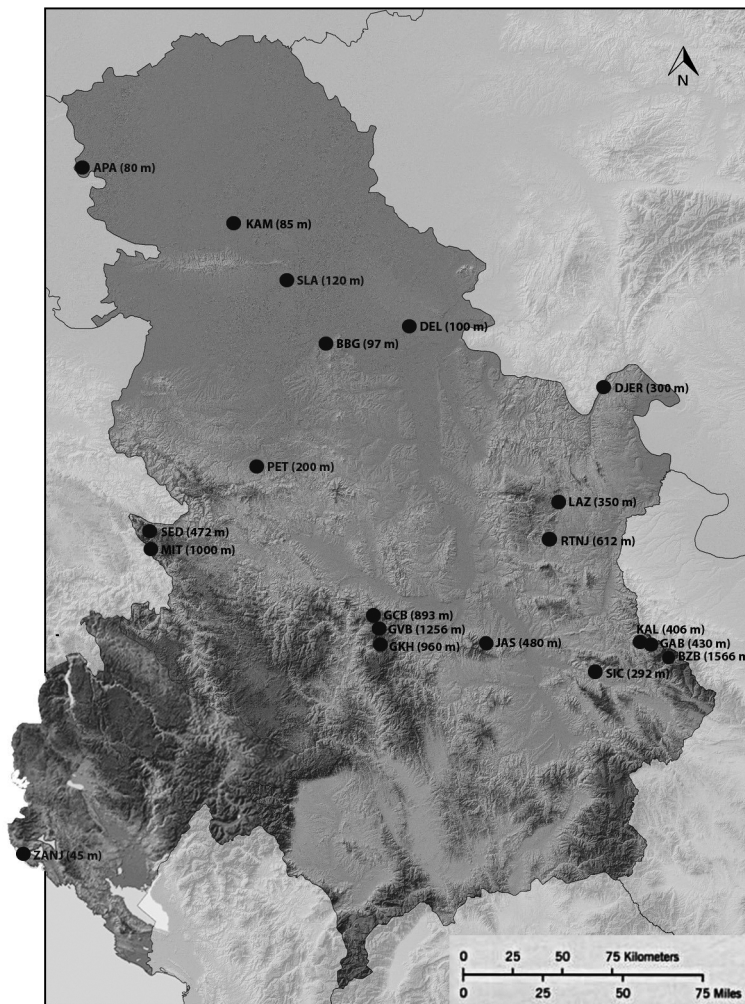
Fig. 1. Localities of *Drosophila subobscura* populations used for the meta-analysis in this paper. APA: Apatin, KAM: Kamariste, SLA: Slankamen, DEL: Deliblato, BBG: Belgrade Botanical garden, DJER: Djerdap, PET: Petnica, LAZ: Lazar river canyon, SED: Sedaljka, RTNJ: Rtanj, MIT: Mitrovac, GCB: Goc1, GVB: Goc3, GKH: Goc2, JAS: Jastrebac, KAL: Kalna, GAB: Gabrovnica, BZB: Babin Zub, SIC: Sicevo, ZANJ: Zanjic. Altitude is given in parentheses. Additional information about the sample sites is provided in Table S1a in Supplement 1, www.int-res.com/articles/suppl/c077p205_supp.pdf

device, so the first environmental dataset had 5 variables. The second environmental dataset (EV2) consisted of 19 standard bioclimatic variables and altitudes (Table S2).

The third dataset (EV3) also represented environmental variables related to temperature and humidity for the period April–June (minimum, average and maximum temperature and minimum and relative average humidity: minT, maxT, avT, Hum_min and Hum_av, respectively), but taken for the particular year of sampling, and locality according to the nearest recording station. These weather parameters were extracted from the online repository of the Republic Hydrometeorological Service of Serbia (www.hidmet.gov.rs). All environmental variables and altitudes were *z*-transformed before further analyses.

Spatial autocorrelation for individual arcsine transformed genetic variables and *z*-transformed environmental variables was accessed with Moran's *I* statistics where pseudo-p-values were found through 1000 Monte Carlo simulations in the R package 'spdep' (v. 0.7-7) (Bivand et al. 2013, Bivand & Piras 2015). The neighborhood contiguity by distance function ('dnearneigh') was used, where *d*1 and *d*2 parameters were set to 0 and 1.732, respectively, and the 'longlat' argument was set to 'NULL.' The *d*2 parameter represents the maximum Euclidean distance along spatial neighbor links. Spatial neighbor links among locations were found with the *k*-nearest neighbor function ('knearneigh'), where *k* was set to 1. Such settings allow each location to have at least 1 neighbor.

2.2. Chromosome preparation and analysis

Analysis of inversion polymorphism was carried out on wild captured *D. subobscura* males or F1 progeny of individual females from natural populations. A minimum of 30 males from each population sample were individually crossed with 3–4 virgin females from the Küsnacht laboratory strain. This strain is homokaryotypic for standard chromosomal arrangements on all 5 acrocentric chromosomes (A_{ST}, J_{ST}, U_{ST}, E_{ST}, and O_{ST}), enabling identification of chromosomes with inversions in the progeny. Salivary glands from third-instar larvae were squashed and chromosomes stained with aceto-orcein solution. For cytological analysis of gene arrangements, the

rather than short-term weather conditions. For the first dataset (EV1), we extracted monthly temperature and precipitation averaged across 30 yr and further averaged the mean precipitation, and the mean, minimum, and maximum temperatures across 3 months (April, May, and June) before sampling. The 3 mo period before sampling was considered in extrapolations to better reflect the averaged environmental conditions at the sampling localities that had an influence, as this time period represents about 4 generations of *D. subobscura* prior to sampling. Along with these variables, we recorded the exact altitudes from a GPS

chromosome map of Kunze-Mühl & Müller (1957) was used. To minimize any error in determining the karyotype of the crossed male, the chromosomes of 8 third-instar larvae were analyzed from the progeny of each cross, and the gene arrangement frequencies were calculated for each population. The sample size of the studied populations, as the number of males or sex chromosomes (which is half the number of autosomes in *D. subobscura*), is given in Table S1.

2.3. Statistical analysis of genetic and environmental data

The Shannon-Wiener diversity index *H'* was used as a measure of diversity of chromosome arrangements among populations to describe the general spatial distribution of inversion variability. *H'* was obtained for each population separately by using the frequencies of all chromosome arrangements together within each population. Linear regression was used to test the correlation between arrangement diversity (*H'*) and longitude, latitude, and altitude.

For all other analyses, linear regressions and principal component analyses (PCAs), chromosomal arrangement frequency data were arcsine (square root) transformed. In order to assess general patterns within both genetic and each environmental dataset, we analyzed the respective matrices with PCA performed in the 'FactoMineR' package v.1.35 (Le et al. 2008) for R v3.4.0 (R Core Team 2017). PCA provides correlations (known as 'correlation loadings') between original variables and principal axes (eigenvectors) along with the significance of the relationship.

The statistical relationship between chromosomal inversions and environmental data was evaluated with Esconfier's RV coefficient and Mantel test using 999 permutations in the 'FactoMineR' package v.1.35 and 'vegan' package v2.4-3 (Oksanen et al. 2017) for R v3.4.0 (R Core Team 2017), respectively. Given that the Mantel test requires that the matrices compared are of the same dimensions, we calculated Euclidian distances among localities (rows in the matrix) defined by genetic data (columns of arcsine-transformed data) and environmental data (columns of *z*-transformed environmental variables). In order to examine joint effects of environmental variables on chromosomal gene arrangement frequencies, we performed a PCA on all *z*-transformed environmental variables, and used principal components (PCs) as individual predictors for further analysis. The effect of each PC on the frequency of any individual chromosomal arrangement was analyzed by linear regression.

Statistical relationships between genetic and environmental data were also determined at 2 levels, treating all chromosomal inversions together as 1 matrix (general association), and separately for each chromosome. Since we did not find significance at any level, we conducted a test to understand if some inversions are related to some of the environmental variables. To do so, we performed a pairwise correlation test among PC scores obtained from separate PCAs of the genetic and respective environmental data. Such an approach is tightly related to multiple factor analysis, and can be interpreted as symmetrical variable selection. This is suitable for the present study, as it does not assume an asymmetrical relationship between the matrices compared, but rather enables a way of finding subspaces in 2 datasets that are related to some degree. However, performing variable selection with a PCA may not be suitable if the relationships between predictors are not linear, and if they do not show clearly distinctive loadings (correlations, in this case) on the PCs, but contribute to the many dimensions uniformly. Therefore, we performed an asymmetrical statistical test, a redundancy analysis (RDA), in order to test if selected environmental variables from a PCA can predict the pattern in the subset of the genetic data (Legendre & Gallagher 2001). To test the effect of individual variables across localities, we performed regressions of each selected inversion (response or dependent data, denoted with *y*) onto each selected environmental variable (explanatory or independent data, denoted with *x*). The length of individual vectors along the RDA axis represents the contribution of the each variable from *x* in explaining the variation in *y*. Statistical significance in linear regressions was obtained with 999 permutations.

3. RESULTS

3.1. Spatial distribution of inversion frequencies and associations among inversions

The frequencies of 31 chromosomal arrangements found in 20 *D. subobscura* populations are given in Table S1. All 5 standard arrangements (A_{st}, J_{st}, U_{st}, E_{st}, O_{st}) and 8 arrangements with inversions (A_1, J_1, U_{1+2}, U_{1+2+6}, E_8, E_{1+2+9}, O_{3+4}, O_{3+4+1}) are present in all populations. Several arrangements are present with low frequency in 9 populations only. Chromosome O is the richest in arrangement diversity, including those with the lowest frequency, such as O_1 and O_{3+4+6}, or O_5 and O_{22}, that are identified in 1 or 2 pop-
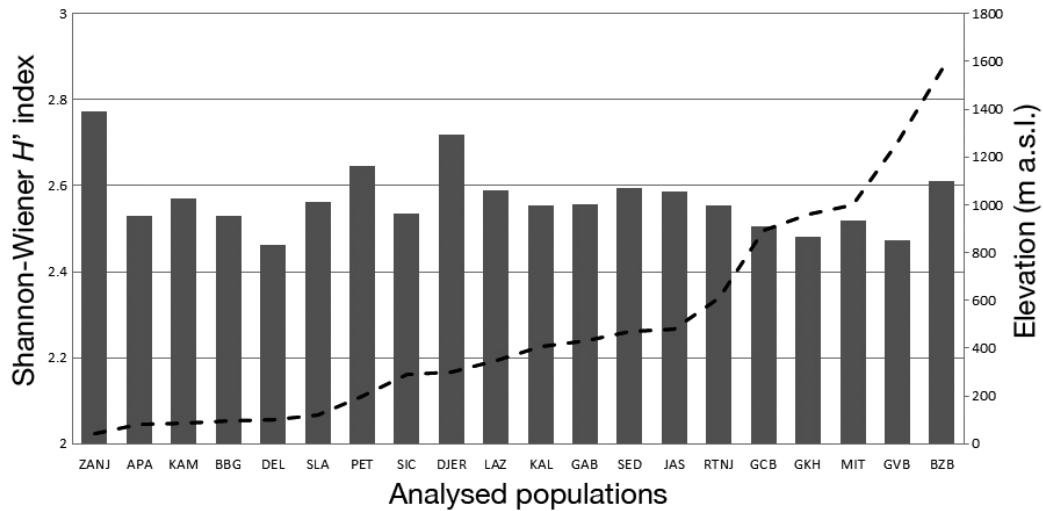
Fig. 2. Shannon-Wiener diversity *H'* index (left *y*-axis) obtained for 20 *Drosophila subobscura* populations (see Fig. 1 for locations). Dotted line represents elevation of the sampling localities (right *y*-axis)

ulations, respectively. The arrangements U_{1+2+7}, J_{3+4}, and A_{2+8+9} are found in 1 population each. The Shannon-Wiener diversity index *H'* as a measure of diversity of chromosome arrangements among populations is given in Fig. 2. No correlation between arrangement diversity with either longitude, latitude, or altitude was obtained.

We conducted a PCA on the arcsine-transformed frequencies of arrangements on all chromosomes for all populations, and the magnitudes of significant correlations between type of inversions and eigenvectors are given in Fig. 3a. Significant correlations were found in PC axes ranging from 1 to 12, indicating that variation in genetic data spans many dimensions. We visualized both the PC coefficients (loadings) for each variable and the PC scores for each observation (Fig. S1 in Supplement 1). PC1 explains 35% of the variation in chromosomal inversions in all populations, while PC2 explains 22.24% and PC3 11.06%. With the exception of Ast, the loading coefficients of PC1 reveal a pattern: a weighted contrast between standard chromosomal gene arrangements (J_{st}, U_{st}, E_{st}, and O_{st}) with negative loading against most of the complex chromosomal gene arrangements with positive loading (E_{1+2}, E_{1+2+9}, U_{1+2}, U_{1+2+6}, O_{3+4}, O_{3+4+1}, O_{3+4+22}). The simple inversion J_1 also shows a relatively high positive loading.

3.2. Association among environmental variables

The magnitudes of significant correlations between original variables of EV1 and eigenvectors from PCA performed on the EV1 dataset are given in Fig. 3b,

and the accompanying PCA plots are given in Fig. S2. Significant correlations were found only for PC1 and PC2. PC1 explains a considerable amount of variation (89.69%) and separates localities with high temperature/lower altitude from those with low temperature/higher altitude. Although the mean precipitation is mostly associated with PC2, altitude and precipitation have positive loadings, while minimum, maximum, and mean temperatures averaged over 3 mo have negative PCA loadings. The distribution of localities shows that the differences among them are primarily due to temperature, not precipitation.

The magnitudes of significant correlations between original variables of EV2 and eigenvectors found in the PCA performed on EV2 are given in Fig. 3d. All PCA plots are given in Fig. S3. The PCs after PC5 did not show any significant correlation with bio-variables. The first PC explains 48.59% of the variation, while the second and third axes explain 36.65 and 8.53%, respectively. PC1 shows a trend whereby precipitation variables (bio18, bio17, and bio14) have positive PC correlation loadings. Those related to temperature (bio5, bio10, bio1, bio6, bio11) have large negative correlation loadings on PC1. PC2 and PC3 were mostly associated with bioclimatic variables that are related to precipitation (see Table S2 for definitions of bio-variables).

The magnitudes of significant correlations between original variables from EV3 and eigenvectors found by the PCA performed on this dataset are shown in Fig. 3c, and all PCA results performed on EV3 are provided in Fig. S4. Significant relations were found only for PC1, PC2, and PC3. PC1 explains a moderate amount of variation (60.25%) and was similar to EV1,
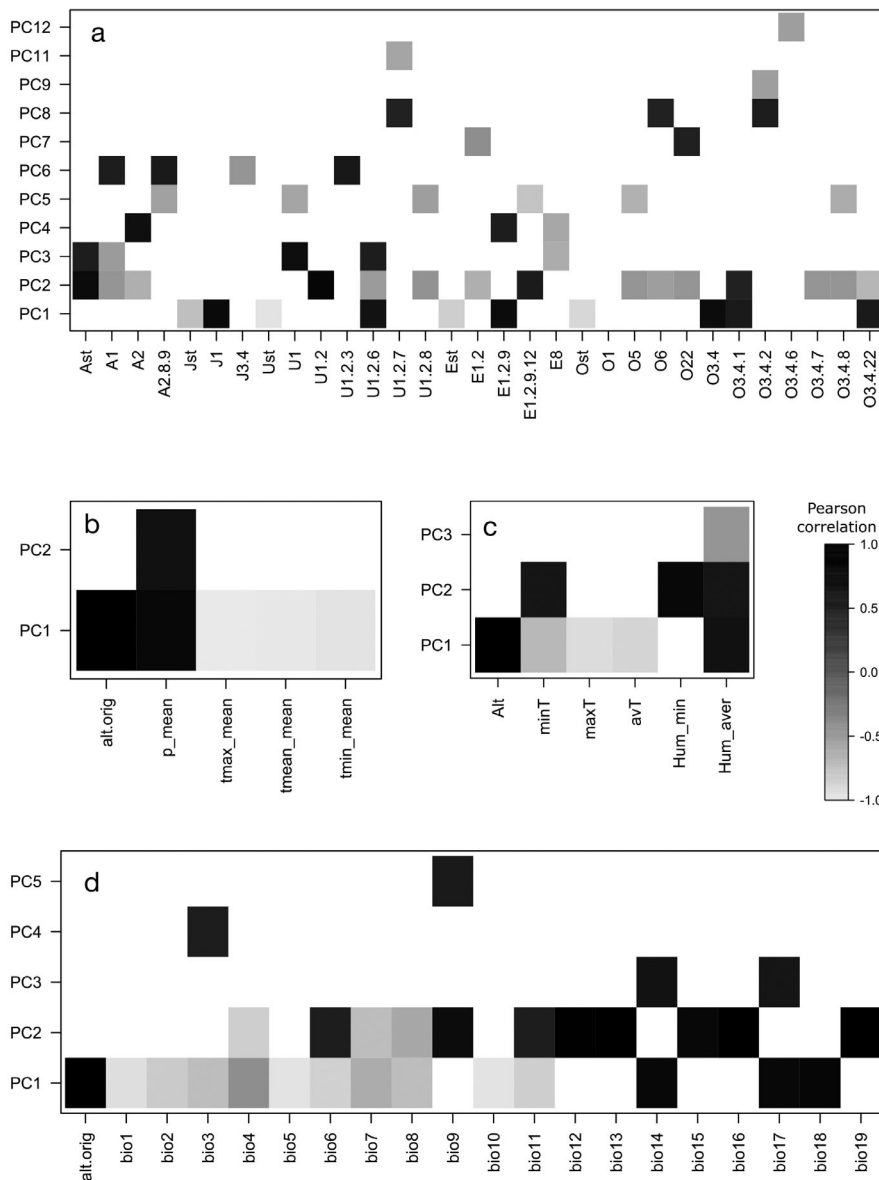
Fig. 3. Significant correlations between original variables and eigenvectors obtained from principal component analysis (PCA) performed on (a) genetic data, and on environmental datasets: (b) EV1, (c) EV3, and (d) EV2. Variables bio 1–19 are defined in Table S2. The dot in gene arrangements means the same as '+' in the body text. Grey scale corresponds to the magnitude of the correlations. Only significant (p < 0.05) correlations are shown. The threshold for significance was 0.05

icantly correlated with 18 of 24 variables, demonstrating the non-independence of predictors. Thirteen out of 18 variables are related to temperature, 4 to precipitation, with altitude as a separate variable. PC2 and PC3 explain 31.47 and 8% of the variance, respectively. Of the 3 PCs, only PC2 shows a significant negative correlation with latitude (r = −0.53, p < 0.01).

3.3. Effects of climatic variables on chromosome arrangement frequencies

If the correlation of the frequencies of individual chromosomal gene arrangements with environmental data PCs is considered, the frequency of A_{st} shows a significant negative correlation with PC2 (r = −0.43, p < 0.05), while E_{1+2} frequency shows a significant positive correlation with both PC1 (r = 0.48, p < 0.05) and PC2 (r = 0.5, p < 0.05). It seems that A_{st} is more frequent at higher altitudes, and therefore has a positive relationship with precipitation values. On the other hand, complex chromosomal arrangements of U, E, and O chromosomes are affected by temperature, particularly the maximum temperature of the warmest month.

According to linear regression analysis of climatic variables against PCs of the inversion frequencies, none of the PCs showed a significant correlation with altitude or latitude. Accordingly, individual climatic variables showed no correlation with inversion PCs.

Effects of EV3 dataset variables on inversion arrangement frequencies were analyzed for their potential intercorrelations by linear regression of inversion frequencies against the environmental variables related to temperature and humidity, as well as against longitude, latitude, and altitude. Of all inversion arrangements on all 5 chromosomes, significant correlations were found for particular arrangements on A, U, and E chromosomes (Table 1).

separating localities with high temperatures/lower altitude from those with low temperatures/higher altitude. PC2 and PC3 were predominantly associated with humidity variables.

We analyzed joint effects of multiple climatic variables (datasets EV1 and EV2) on inversion frequencies for their potential intercorrelations (multicollinearity). The obtained PC1 explains 54.85% of the variance in the overall climate data, and is signif-

Table 1. Regression coefficients (r) of chromosomal inversion frequencies against environmental variables (dataset EV3). Only significant results (p < 0.05) are shown. Hum_av: average humidity, minT: minimum temperature

Chromosomal arrangement	EV3	r	p
A_{st}	Hum_av	0.548	0.012
E_{1+2}	minT	0.501	0.024
	Altitude	−0.516	0.019
E_{1+2+9}	Altitude	0.465	0.038
	Latitude	−0.44	0.05
U_{1+2+6}	Hum_av	0.489	0.028

Table 2. Association between 2 matrices: genetic data (overall and within each chromosome) and environmental data (datasets EV1, EV2, EV3) inferred by the RV coefficient and a Mantel test (r). Significance was found by 999 permutations. A, J, U, E, and O are chromosomes

	Environmental dataset	RV	p	r	p
Overall	EV1	0.16	0.264	0.1	0.202
	EV2	0.22	0.265	0.13	0.184
	EV3	0.21	0.247	0.09	0.267
A	EV1	0.09	0.311	0.03	0.348
	EV2	0.19	0.125	0.19	0.135
	EV3	0.15	0.206	0.17	0.109
J	EV1	0.09	0.311	0.03	0.345
	EV2	0.19	0.125	0.19	0.131
	EV3	0.15	0.206	0.17	0.108
U	EV1	0.08	0.473	0.04	0.286
	EV2	0.13	0.517	0.04	0.351
	EV3	0.14	0.385	0.06	0.318
E	EV1	0.2	0.08	0.09	0.219
	EV2	0.24	0.126	0.15	0.149
	EV3	0.25	0.07	0.04	0.379
O	EV1	0.11	0.354	−0.01	0.504
	EV2	0.17	0.338	0.06	0.308
	EV3	0.15	0.379	−0.05	0.654

According to the RV coefficient and the Mantel test, the relationship between environmental data both overall and within chromosome genetic variability is not statistically significant (Table 2). This was confirmed in a test which compared pairwise correlations between PC scores obtained from separate PCAs of genetic and environmental data (Fig. 4). Specifically, it is notable that most leading PCs of genetic and environmental matrices are not related to each other, suggesting that patterns of inversions and environmental variables are not generally associated (Tables S3 & S4). However, lower dimensions showed some degree of correspondence, and it turned out that site scores along PC4 of inversion
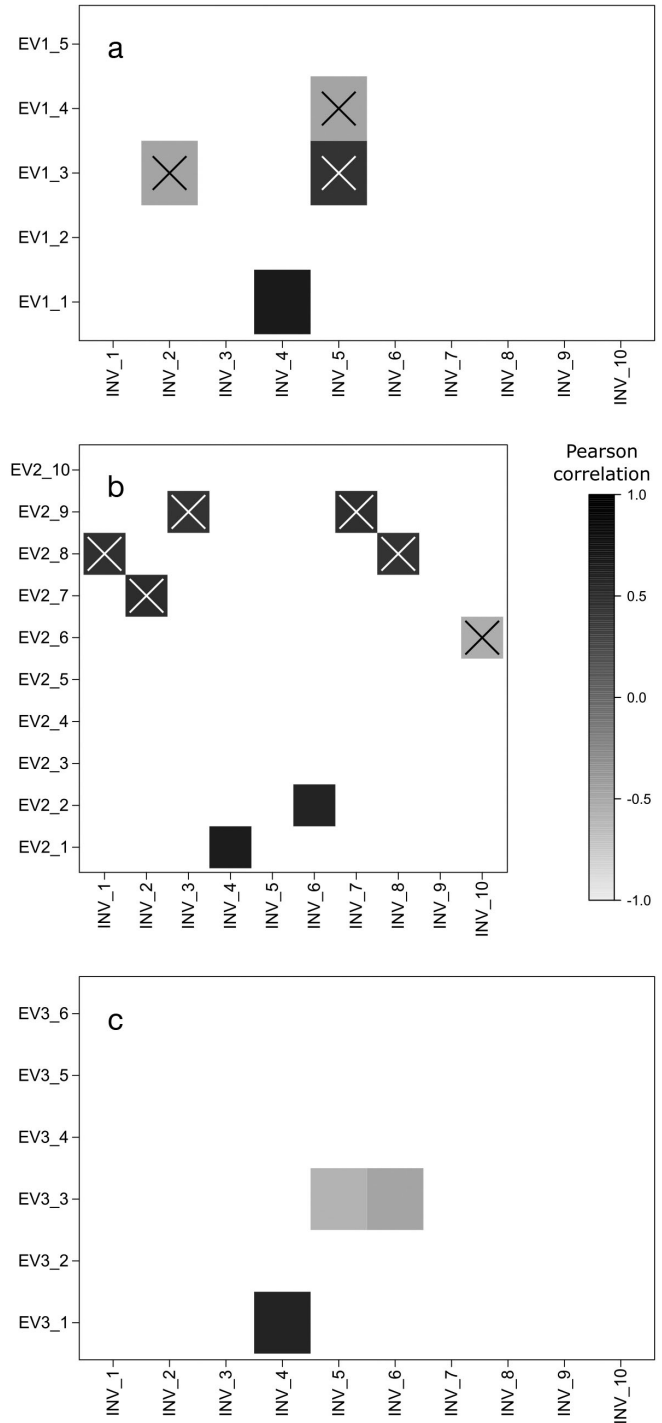

Fig. 4. Pairwise correlation among principal component (PC) scores obtained from separate PCAs performed on genetic data (INV) and environmental datasets: (a) EV1, (b) EV2, and (c) EV3. Significance was estimated through 999 permutations, and the threshold for significance was 0.05. Grey scale corresponds to the magnitude of the correlations. Numbers after the underscore on each tick label refer to the respective PC axis. Only significant (p < 0.05) correlations are presented. Axes that lack significant load of original variables are marked with a cross
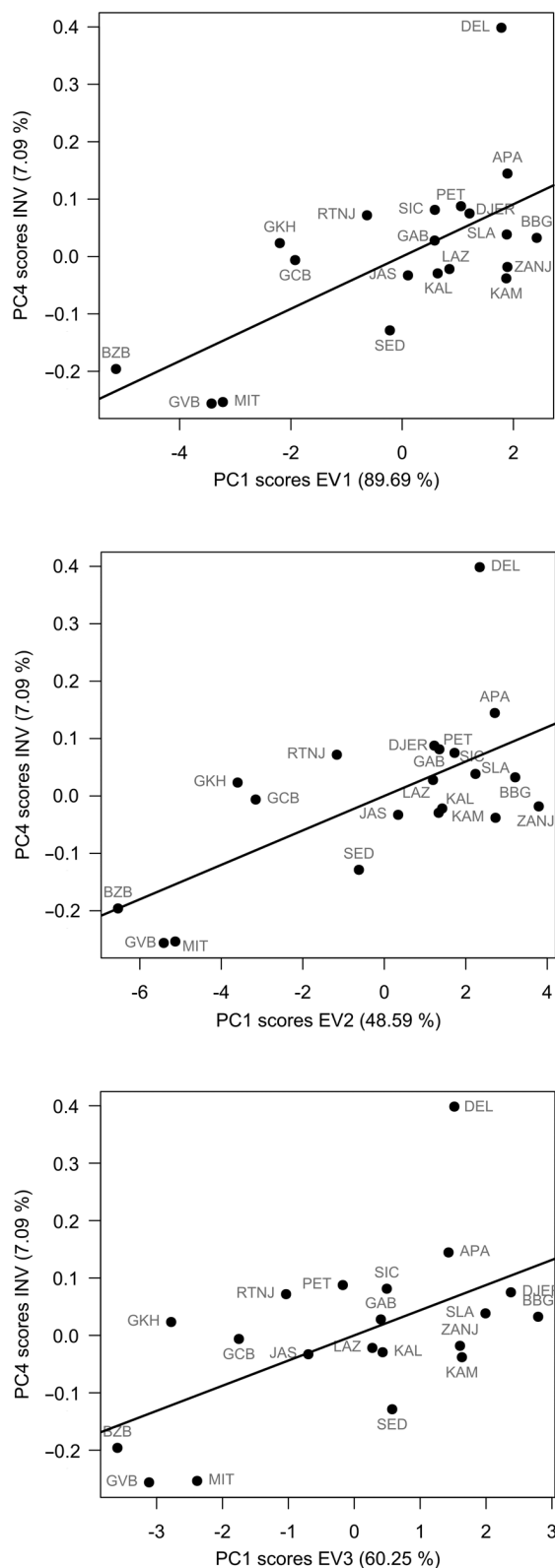
Fig. 5. Linear relationship between PC4 scores of the inversion dataset and PC1 scores of environmental data (datasets EV1, EV2, and EV3). Site abbreviations as in Fig. 1

data and site scores along PC1 of different environmental data were significantly correlated (Fig. 5). Inversions that are positively correlated with PC4 were A_2 and E_{1+2+9}, whereas E_8 showed a negative correlation with the same PC. Likewise, variables associated with PC1 of the environmental data were generally structured in a way that temperature variables were negatively correlated with PC1, whereas positive correlations were observed for altitude and precipitation. Most other significant associations were observed for higher-ordered PCs, and they are not relevant to the conclusion, as none of the original variables was significantly associated with them (e.g. none of the subsequent PCs after PC2 and PC5 of EV1 and EV2, respectively, was strongly associated with any original variables).

RDA was used to test if selected environmental variables predict a pattern in a subset of genetic data (Table 3). Two models that compare PC6 from inversion data (INV_6) with environmental data EV2 and EV3 produced significant results. Inversions associated with the INV_6 axis were A_1, A_{2+8+9}, J_{3+4}, and U_{1+2+3}. However, apart from A_1, all other inversions showed very low variance, and therefore are not relevant. Likewise, the results of RDA that compared INV_6 versus EV2 revealed a non-significant relation when we excluded the ZANJ population, an outlier according to a graphical inspection. In general, the results indicate that selected environmental variables cannot predict the pattern of the selected genetic variables. When each inversion and environmental variable were taken as dependent and independent variables, respectively, almost all linear regressions returned a non-significant result (Supplement 2, www.int-res.com/articles/suppl/c077p205 _supp.xls). Only arrangements E_{1+2+9}, A_1, and U_{1+2+8} were significant with regard to environmental data.

As shown earlier, the INV_4 PC was significantly correlated with arrangements E_8, E_{1+2+9}, and A_2. Almost all individual regression results showed non-significance, but after excluding an outlier (MIT), the inversion E_{1+2+9} appeared positively associated with precipitation variables and altitude, and negatively associated with temperature. RDA analysis showed that only 2 chromosomal inversions are to some degree related to environmental data. This analysis serves only to summarize the relationship between E_{1+2+9} inversion and other variables, and cannot predict an E_{1+2+9} pattern, as the RDA model showed non-significance ($F = 1.04$, $p = 0.501$). An informative result was the A_1 inversion which was positively associated with bio9 (mean temperature of the driest quarter) and bio15 (precipitation seasonality) and

Table 3. Redundancy analyses (RDA statistics). Dependent variables are inversions correlated with PC axes of genetic data (INV), while predictor variables are environmental variables correlated with PC axes of environmental data (datasets EV1, EV2, EV3). The number after the underscore refers to particular PC axis. Other abbreviations are as used throughout the article and in Table S2. Significant values are marked with an asterisk (*); italics: results when population ZANJ (see Fig. 1) is excluded as an outlier

PCA axis	% of explained variance	Variables correlated with PC axis	Type of variables in RDA	df_1	df_2	F	R^2	R^2 adj.	p
INV_4	7.09	E_8, E_{1+2+9}, A_2	Dependent	5	14	1.04	0.27	0.01	0.445
EV1_1	89.69	tmax_mean, tmean_mean, tmin_mean, p_mean, altitude	Predictors						
INV_4	7.09	E_8, E_{1+2+9}, A_2	Dependent	13	6	0.91	0.66	−0.07	0.604
EV2_1	48.59	bio1-8, bio10-11, bio14, bio17-18, altitude	Predictors						
INV_6	4.32	U_{1+2+3}, A_{2+8+9}, A_1, J_{3+4}	Dependent	11	8	2.73	0.79	0.5	0.029*
EV2_2	36.65	bio4, bio6-9, bio11-13, bio15-16, bio19	Predictors	*11*	*7*	*1.45*	*0.69*	*0.22*	*0.261*
INV_4	7.09	E_8, E_{1+2+9}, A_2	Dependent	5	14	1.09	0.28	0.02	0.387
EV3_1	60.25	maxT, avT, minT, Hum_aver, altitude	Predictors						
INV_5	6.17	$E_{1+2+9+12}$, O_5, O_{3+4+8}, U_1, U_{1+2+8}, A_{2+8+9}	Dependent	1	18	1.42	0.07	0.02	0.254
EV3_3	7.5	Hum_aver	Predictors						
INV_6	4.32	U_{1+2+3}, A_{2+8+9}, A_1, J_{3+4}	Dependent	1	18	6.12	0.25	0.21	0.005*
EV3_3	7.5	Hum_aver	Predictors						

negatively correlated with average humidity ($F = 5.57$, $p = 0.002$). Also, the arrangement U_{1+2+8} was significantly associated with humidity from the EV3 dataset, but exhibited low variance. Only flies from localities ZANJ, DJER, and SED had this type of inversion.

Spatial autocorrelation analysis for individual arcsine-transformed genetic data showed that only 3 out of 31 variables were spatially autocorrelated (A_{2+8+9}, $p = 0.03$; U_1, $p = 0.015$; $U_{1+2} = 0.03$), and for *z*-transformed environmental variables EV1, 2 out of 5 variables were autocorrelated (altitude, $p = 0.04$; p_mean, 0.001), 8 out of 19 for EV2 (bio4, $p = 0.021$; bio12, $p = 0.001$; bio13, $p = 0.032$; bio14, $p = 0.009$; bio16, $p = 0.025$; bio17, $p = 0.001$; bio18, $p = 0.014$; bio19, $p = 0.001$), and none in the EV3 dataset. Overall, only 21.7% (13 out of 60 variables) of the data were autocorrelated while 78.3% were not. We therefore decided to perform all other analyses without taking autocorrelation into account.

4. DISCUSSION

Long-term changes in chromosomal inversion polymorphism in *Drosophila* species suggest that the genetic constitution of populations is responding to climate change (Van Heerwaarden & Hoffmann 2007). The present study is a comprehensive meta-analysis of previously published and unpublished data of inversion polymorphism of 20 *D. subobscura* populations from the Central Balkans (19 from Serbia, 1 from the Montenegro coast) using different environmental data in the context of variability of climatic parameters.

The climate of the region can be classified as a warm-humid continental or humid subtropical. Differences in elevation, proximity of the Adriatic Sea, Mediterranean Sea, and large river basins, as well as exposure to winds, account for climate variations. This region is characterized by extensive ecological and landscape diversity, and is considered one of the main European refugia for many species during the last 2 million years of glacial/interglacial climate oscillations (Hewitt 2004). Global and microclimatic changes affect the habitats in this region and genetic polymorphisms of *D. subobscura* populations represent an attractive and informative model in studying environmental interaction with genetic variability.

Latitudinal inversion clines like those obtained for *D. melanogaster* along the Australian coast are much simpler, as there is no variation from altitude or sea proximity (Knibb 1982, Anderson et al. 2005). The clinal distribution of chromosomal variability in the New World populations of *D. subobscura* mirrors those found in populations from Europe, and studies indicate that inversion polymorphism in this species is primarily sensitive to temperature changes (Rodríguez-Trelles & Rodríguez 1998, Balanyá et al. 2003, Rodríguez-Trelles et al. 2013).The results presented here show that frequencies of most chromosomal arrangements in *D. subobscura* change along lati-

tude, with standard chromosomal arrangements being associated with cooler environments, whereas more complex arrangements appear 'warm-adapted'. This general pattern is reflected even in the samples from this narrow latitudinal range. In *D. subobscura* populations from southwestern and central Europe studied to date, an increase in the frequency of chromosomal arrangements typical to southern latitudes, and a decrease in the frequency of chromosomal inversions typical to northern latitudes, have been observed, and long-term studies over the years have shown a significant shift in the gradient of southern arrangements northwards (Orengo & Prevosti 1996, Rodríguez-Trelles & Rodríguez 1998, Solé et al. 2002, Balanyá et al. 2006, 2009, Zivanovic & Mestres 2011).

In the present study, we included climate parameters from 2 databases (World Clim and Republic Hydrometeorological Service of Serbia) averaged over 3 mo before the sampling, as relevant for the study. The chosen months represent a critical seasonal period that affects the population structure, effective population size, distribution, migration, selection pressure and development of *D. subobscura* before sampling in late June. The general agreement obtained in the results, whether EV1 or EV3 datasets were used, supports using such an approach in studying associations between genetic and environmental variables. Our previous studies, and most of the related studies of *D. subobscura* inversion polymorphism as a potential indicator of climate warming, were focused on calendar data of temperature and humidity matching the days of sampling, which rather reflect adaptive plasticity than a response to climate change. As suggested by Rodríguez-Trelles & Rodriguez (2007), using climatological and biological dates better reflects a possible impact of global warming, which has generally prolonged the growing season at higher latitudes. A study performed on 2 populations from Goc Mountain over 4 yr showed a significant decrease in the frequency of chromosomal arrangements on O and U chromosomes, which are otherwise characteristic for northern latitude (Andjelkovic et al. 2007). The present meta-analysis, with 3 sets of environmental variables, did not show a significant correlation between the O chromosome arrangement frequencies and temperature.

Seasonal cycling of chromosomal inversions has been recorded in a number of *Drosophila* species (for review, see Rezende et al. 2010). Genes located within inversions are associated with a variety of traits, including those involved in thermal adaptation (Hoffmann & Rieseberg 2008). Of the 5 acrocentric chromosomes in *D. subobscura*, the O chromosome

(homologous to the 3R chromosomal arm of *D. melanogaster* and the second chromosome of *D. pseudoobscura*) are the longest and most polymorphic, with about 40 inversion arrangements (Krimbas 1993). Inversion arrangements on the O chromosome, such as O_{3+4}, appeared indicative of associations with heat-tolerance and thermal adaptation (Laayouni et al. 2007), and functionally relevant genes are located in this arrangement. The frequency of O_{3+4+7} increased 5 times from the average after an early spring heat wave in 2011 in 2 *D. subobscura* populations from Spain, but returned to the usual seasonal frequency afterwards (Rodríguez-Trelles et al. 2013). This species shows adaptive plasticity through reversible responses to a fluctuating temperature, and some arrangements, like those on the O chromosome, are indicative genetic markers of such immediate responses.

Temperature is often thought to be the dominant factor in shaping clines due to its tight relationship with latitude. However, north–south clines in *D. subobscura* are best explained by a combination of climatic variables, suggesting that the selective agent is not related to temperature itself (Menozzi & Krimbas 1992, Krimbas 1993). This is confirmed in thermal adaptation experiments with 'warm' and 'cold' lines in *D. subobscura*, which gave an inconsistent replicate of the clinal pattern observed in natural population patterns (Santos 2007). The results in the present paper indicate that arrangement frequency patterns are likely driven by a synergistic effect of factors related to temperature and precipitation. At the same time, they illustrate the difficulty of isolating potentially causal selection factors by statistical analysis alone. A closer look at the loadings of PC1 suggests that it is overall positively correlated with temperature, while negatively correlated with precipitation. Thus, the frequencies of standard chromosomal arrangements tend to covary positively with precipitation, whereas parameters related to temperature appear to favor higher frequencies of the inverted and more complex chromosomal arrangements. If precipitation variation increases, the frequency of A_1 increases as well, whereas it tends to decrease with increasing humidity. A complex relationship among local environmental variables is observable from the results that reflect the probable effect of altitudinal shifts, because altitude is negatively correlated with temperature, and positively correlated with precipitation. The altitudinal gradient of inversions is different from their latitudinal gradient, and the frequency distribution pattern does not confirm the hypothesis

that higher altitudes match ecologically marginal conditions for *D. subobscura* (Burla et al. 1986).

The effects of global climate change on populations of different species combine with the local environmental specificities. Local adaptations, facilitated by inversion polymorphism, are hypothesized to cause broad distributions of insect species such as *Anopheles gambiae* (Rocca et al. 2009) and suggested earlier for *D. subobscura* (Jelić et al. 2009, 2012). Owing to the overall low density of the available climate stations, not all variation is captured, particularly of precipitation that may occur at a spatial resolution of 1 km in some areas. The highest uncertainties in climate data are in the areas with high variation in elevation, like gorges, canyons, and mountains. Three of the samples in this paper are from gorges (DJE, SED, SIC), 1 is from a canyon (LAZ), and 3 (MIT, BZB, GVB) are from mountains over 1000 m altitude. They all feature contrasting temperature data, humidity, and light along the slopes. In cases of mountain localities, discrepancies exist between altitude data from the WorldClim base and our original data. Elevation bias is present in the station network used, but it is unclear how this affects the results (Hijmans & van Etten 2012, Fick & Hijmans 2017). Our analysis points out how outliers (such as ZANJ from the sea coast or MIT from the mountain locations) can affect the findings, as they can mask the pattern, but also exaggerate it, thus leading to false positive conclusions. For instance, after excluding MIT, the E_{1+2+9} inversion is positively associated with precipitation variables and altitude, and negatively with temperature.

Our results suggest that the pattern of inversions is generally not associated with environmental variables, and a particular inversion cannot be a predictive genetic marker of global climate change. Populations in different habitats are subjected to habitat-specific selection regimes, and other factors, such as gene flow and genetic drift, can complicate distinguishing selective from other sources of the observed genetic variability pattern. The inversion polymorphism data of *D. subobscura* suggest that the Balkan Peninsula may have played an important role in repopulating Europe after the last glaciation (Krimbas 1993). Genetic diversity among 3 *D. subobscura* populations (2 from gorges [SED, SIC] and 1 from a canyon [LAZ]) included in this meta-analysis was studied earlier by integrating data from multiple genetic markers (chromosomal inversions, mtDNA, and microsatellite variability) to reveal their present genetic structure and possibly deduce the historical processes that shaped it (Jelić et al.

2012, Stamenkovic-Radak et al. 2012). Two of those populations are recognized as refugia in terms of their floristic and faunistic data, and their specific natural habitats are probably shaped by glacial–interglacial climate oscillations. They have some rare gene arrangements with low frequency and low variance, such as U_{1+2+8}, which appeared in only 3 populations, and is significantly associated with humidity.

D. subobscura is a temperate species, adapted to large fluctuations in environmental factors such as temperature and humidity, but with local effects of genetic drift that are overcome with high gene flow in this species. The result of an experimental study of the adaptive potential of inversion polymorphism in this species (Fragata et al. 2014) has shown that selection is shaping the evolutionary response of inversion frequencies in varying environmental conditions, albeit within constraints from the previous population history. Populations of *D. subobscura* are rich in inversion gene arrangements, but have different potentials to adapt and overcome environmental changes. The inversion polymorphism of this species is not rigid, but rather is considered semi-flexible, and particular arrangements (like on the O chromosome) enable immediate adaptive plastic responses to thermal stress imposed by global climate change. In the long term, gene arrangements on other chromosomes (like A, E, and U) enable adaptation to a particular combination of ecological parameters in each population. The synergistic effects of these factors determine population-specific and species-specific responses to global and local environmental changes.

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