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Local adaptation at fine spatial scale through chromosomal inversions and mito-nuclear epistasis: Findings in *Drosophila subobscura* (Diptera: Drosophilidae)

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Abstract. To explore local adaptation in wild populations at a fine spatial scale we characterized the genetic variability of eight closely located populations of *Drosophila subobscura* and its associations with microhabitat environmental conditions. Three different genetic markers were assessed: chromosomal inversions, a SNP of mitochondrial *ND5* gene and nuclear microsatellites. Population genetic analyses of chromosomal variability revealed significant genetic differentiation between these populations. Gene arrangement frequencies on the E chromosome contributed most to these differences. We also investigated role of mito-nuclear epistasis in mitochondrial genome differentiation and revealed weak linkage disequilibrium (LD) exclusively between O_{3+4} inversion arrangement and mitochondrial DNA haplotype I in two populations. In addition, the trend in the LD between O_{ST} chromosomal arrangement and haplotype II was general in the total sample. Microsatellite analysis revealed an absence of stochastic processes, like census reduction, upon population differentiation. Only a small amount of the genetic variation is related to geographic distance, while most (97%) is attributable to other factors and in some degree to microhabitat variables (temperature, humidity). The analysis of these factors revealed they effect inversion arrangement frequencies, especially E_{1+2+9}, E_{ST} and O_{ST}. Even though this model organism is known for its high mobility and mostly large effective population size, the results presented here reveal that local adaptations can occur even at a small spatial scale. We propose that locally adapted alleles within chromosomal inversions, as well as joint selective pressures acting on mitochondrial and nuclear genomes, are responsible for the observed adaptation to microhabitat conditions.

INTRODUCTION

Local adaptation can be defined as that which results in populations of a species being fittest in their respective local environments (Kawecki & Ebert, 2004). It is theoretically assumed to result from: population differentiation within a meta-population, divergent natural selection in local habitats and environmental heterogeneity in local habitats in combination with low dispersion and gene flow among populations (Tiffin & Ross-Ibarra, 2014). Integrative population genetic approaches are widely used for investigating various aspects of local adaptation, including genome variability, the variability of environmental factors influencing genetic variability and spatial distributions of populations (Tiffin & Ross-Ibarra, 2014). The present trend is to combine standard genome-wide analyses and high-resolution searches for candidate genes that are subject to selection and responsible for local adaptations (Savolainen et al., 2013; and references therein).

However, such searches could be generally restricted to coding regions and dependent on annotation information, largely derived from mutational screening of model species in laboratory environments. Thus, they could potentially miss genes with phenotypic effects that differ between laboratory and natural environments, or have a minor or overestimated phenotypic effects (Pavlidis et al., 2012; Tiffin & Ross-Ibarra, 2014). Furthermore, many candidate genes and their phenotypic effects have been studied independently, but most ecologically important traits are determined by many interacting loci (Rockman, 2012).

In addition to finding genes involved in local adaptation, a population genetic approach can be used to identify ecological variables that may drive adaptation by habitat selection, manifested in the clustering of individuals of particular genotypes in habitats where they are fittest (Rosenzweig, 1991). From a population genetics perspective, it is important to elucidate relationships between habitat selection and the genetic variability of populations (Levene, 1953; Maynard Smith, 1966; Hoekstra et al., 1985), particularly given the large body of theoretical work indicating how genetic variation can be maintained in heterogeneous environments (Felsenstein, 1976; Hedrick, 1976) by both temporal and spatial variation in selection coefficients.

In most studies on local adaptations it is implicitly or explicitly assumed that a requirement for its evolution is sufficient geographical distance between populations (Nachman et al., 2003) for selective pressures in particular microhabitats to exceed the homogenizing effects of gene flow (Wright, 1969; Felsenstein, 1976; Garcia-Ramos & Kirkpatrick, 1997; Hendry et al., 2001; Kawecki & Ebert, 2004). This assumption is supported by various empirical findings (Nosil & Crespi, 2004). However, Richardson & Urban (2013) suggest that some studies seriously challenge the presumption that local adaptation occurs only at large geographical scales (Steiner & Berrang, 1990; Kavanagh et al., 2010; Willi & Hoffmann, 2012; Richardson et al., 2014). There is, therefore, accumulating evidence that we should revise theoretical presumptions of local adaptation in terms of spatial scale.

Drosophila subobscura is a highly suitable model species for investigating local adaptation using an integrative population genetic approach. Robust and abundant literature is available on this species' population genetics, and many genetic markers have been developed for its population assessment. First of all, it has a rich diversity of inversion polymorphisms, which is associated, to varying degrees, with dynamic abiotic factors both spatially and temporally, and has clear clinal distributions (Krimbas & Powell, 1992; Živanović et al., 1995; Orengo & Prevosti, 1996; Andjelković et al., 2003; Balanyà et al., 2004; Stamenkovic-Radak et al., 2008, 2012). Therefore, the inversion polymorphism is important in adaptive processes. Several studies have focused on chromosomal variability of two populations of *D. subobscura* from locations close to each other: two environmentally different types of forest habitats on Mount Goč (Central Serbia). These stud-

ies have shown differences in chromosomal arrangement frequencies (Andjelković et al., 2003, 2007; Savković et al., 2004; Stamenkovic-Radak et al., 2008, 2012; Jelić et al., 2009). Stamenkovic-Radak et al. (2008) report the effective population size and chromosomal variation in these nearby populations in repeated samples over several years. They conclude that reduction in population size might have an effect on their genetic differentiation, but they did not address the putative role of local adaptation.

In addition to chromosomal inversion polymorphisms, we also focus here on mtDNA, which in *D. subobscura* is known to have two prevalent haplotypes (I and II). It has a wide geographic homogeneity with two haplotypes, which exist together with the rare ones derived from the two common ones (Afonso et al., 1990; Castro et al., 1999; Stamenkovic-Radak et al., 2012). Seasonal bottlenecks and population expansion are responsible for the excess of singleton mutations (Castro et al., 2010; Christie et al., 2010). Considering the two most frequent haplotypes, selection possibly acts jointly on combinations of mitochondrial and nuclear alleles, and several studies have analysed the linkage disequilibrium (LD) between mitochondrial haplotypes and chromosomal inversions (Oliver et al., 2002; Jelić et al., 2012a, b). These studies show transient LD due to temporal and spatial differences in the action of ecologically specific selection pressures. Balancing selection such as negative frequency dependent selection may also influence the frequencies of the two main haplotypes (Arnqvist et al., 2016).

The third presumably neutral genetic marker considered here are microsatellites (Pascual et al., 2000, 2001). The Palearctic populations are not genetically differentiated geographically based on microsatellites due to the high gene flow among them. Variability of these markers is relevant to this study because it can show possible effect of neutral processes, such as bottlenecks, on the differentiation between local populations.

Based on the previous results, the objective of the present study was to shed more light on local adaptation at a fine geographical scale in the wild using highly mobile model organism *D. subobscura*. Accordingly, we genotyped individuals in eight closely positioned microhabitats, using three genetic markers with different adaptive significances: inversion polymorphism, RFLP of mitochondrial *ND5* gene and microsatellites. Genotyping was complemented with environmental factors (temperature, humidity) and altitude. We address the question whether in situ environmental factors can lead to genetic differentiation between closely located populations, and re-evaluate the theory on the spatial scale required for local adaptation in natural environments.

MATERIAL AND METHODS

Population samples and microhabitat characteristics at the localities sampled

Drosophila subobscura populations were sampled at eight nearby localities (< 15 km) (designated B, BR, VB, PR, H, KH, BZ and NB) on Mount Goč, Central Serbia, in early summer. Their latitude, longitude and altitude are presented in Table 1.

Table 1. Geographical and vegetation descriptions of the localities sampled.

Locality	N	E	Altitude (m)	Exposition	Phytocenosis
B	43°33′29.1″	20°45′17.4″	893	NE	*Abieti-fagetumpauperum*
BR	43°32′32.5″	20°46′32″	1111	SW	*Abieti-fagetumtypicum*
VB	43°32′33″	20°47′10″	1256	NW	*Abieti-fagetumpauperum*
PR	43°32′54″	20°47′11.3″	1170	NW	*Abieti-fagetumpauperum*
H	43°33′05.7″	20°40′13.7″	787	NW	*Ostryo-Quercetumdaleschampii*
KH	43°33′27.4″	20°39′07″	960	E	*Quercetummontanum*
BZ	43°34′04″	20°40′09″	770	E	*Ostryo-Quercetumdaleschampiiserpentinicum*
NB	43°33′32″	20°39′59″	667	SE	*Fagetummoesiacaemontanum*

Phytocenological data were provided by the Faculty of Forestry, University of Belgrade, which governs forests on Goč Mountain. These localities represented microhabitats with different vegetation and local microclimatic conditions. Fig. 1 shows the positions of the sites sampled and their relative difference in recorded temperature and humidity.

Nine traps containing fermenting fruit were distributed and mini-data loggers (Testo-174T, Testo AG, Lenzkirch, Germany) were placed centrally at each locality to record temperature and relative humidity simultaneously with population sampling. The accuracy of the data loggers' temperature and relative humidity recordings are ± 0.5°C (within the range –30 to +70°C) and ± 3% (within the range 2 to 98 %), respectively. The mean temperature, relative humidity and their coefficients of variation at each locality were determined for full-day recordings over four days (24 h recordings), Table 2.

D. subobscura females were collected at each of the eight localities and individually placed in vials used to establish a total of 388 isofemale (IF) lines: 51, 55, 50, 43, 46, 53, 50 and 40 IF lines derived from females collected at the B, BR, VB, PR, H, KH, BZ and NB localities, respectively. All IF lines were reared under constant laboratory conditions (temperature 19°C, RH ~60%, light 300 lux and 12L : 12D cycle) for one generation and one F_1 male from each IF line was used for genetic analysis (focusing on the variability of inversion gene arrangement frequencies, distributions of frequencies of the two most frequent mtDNA haplotypes in *D. subobscura*: I and II, and microsatellite variability).

Genetic variability assessment and data analysis

Inversion polymorphism

One F_1 male from each IF line was individually crossed with three virgin females from the Küsnacht laboratory strain (homokaryotypic for all five acrocentric chromosomes of the

Fig. 1. Map showing the positions of the sites sampled. Mean temperature and relative humidity for each population (B, BR, VB, PR, H, KH, BZ, NB) are labelled using circle size and colour.

species: A_{SP}, J_{SP}, U_{SP}, E_{SP}, O_{ST}). Salivary glands from third-instar larvae were squashed and chromosomes were stained with aceto–orcein solution. To minimize errors in karyotype determinations, eight larvae from the progeny of each cross were analysed. For the cytological analysis of chromosome arrangements, the chromosome map of Kunze-Mühl & Müller (1958) and nomenclature according to Kunze-Mühl & Sperlich (1955) and Krimbas & Powell (1992) were used.

Differences in individual chromosome arrangement frequencies between pairs of populations were evaluated using the Z-test. Sequential Bonferroni correction (Rice, 1989) was used to adjust significance values to account for false positives arising from multiple simultaneous testing. Furthermore, the genetic differentiation in chromosomal polymorphism between populations was gauged by estimating F_{ST} values, based on differences in chromosome arrangements frequencies, using Arlequin software (v 3.5.1.2) (Excoffier & Lischer, 2010).

MtDNA variability

DNA was extracted following Martinez et al. (1992) but without the final alkaline lysis treatment. A 984 bp long region of the mitochondrial *ND5* gene was amplified using previously described primers and PCR conditions (Garcia-Martinez et al., 1998). The amplified fragment was digested with FastDigest *Bsu*RI (*Hae*III) restriction enzyme (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's recommendations. The digested mtDNA fragments were separated on horizontal 1.5% gels containing ethidium bromide (0.1 µg/mL). Gene Ruler DNA 100 bp (Thermo Fisher Scientific) was used as a size standard. After electrophoresis, gels were photographed using a Bio-Rad Gel Doc 1000 fluorescence imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The *ND5* gene has a polymorphic site that differentiates haplotype I (and rare haplotypes descending from it), from haplotype II (and rare haplotypes descending from it). A *Hae*III restriction site is present in haplotype I, but not in haplotype II. Thus, following *Hae*III digestion ND5 fragments from haplotype I yield two (~114 and ~870 bp) bands on a gel, while fragments from haplotype II yield just one band. Distribution of haplotypes was determined for each population. Genetic distances in haplotype diversity between pairs of populations were estimated using the F_{ST} statistics, with modification for a haploid data set as heterozygosity data are not available. Arlequin (v 3.5.1.2) software (Excoffier & Lischer, 2010) was used for these analyses.

Analysis of linkage disequilibrium (LD) between mtDNA and inversion polymorphism

Linkage disequilibrium among mitochondrial haplotypes and chromosomal arrangements was tested separately in each population, and for the whole sample set according to Jelić et al. (2012a, b) and Oliver et al. (2002). *D* and *D′* values were estimated (Lewontin, 1964). In order to test the significance of LD, these calculations were followed by Fisher's exact test for independ-
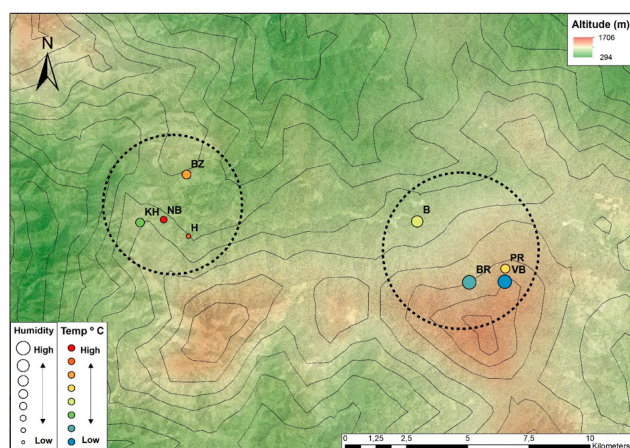
Table 2. Temperature (T) and relative humidity (H) at the localities sampled.

Locality	B	BR	VB	PR	H	KH	BZ	NB
T (min–max, °C)	8.6–17.9	6.8–16	5.5–15.9	9–19.9	11–28.8	8.9–20.4	11.5–18.9	10.5–28.8
Mean T ± SE (°C)	13.3 ± 0.2	10.0 ± 0.1	9.1 ± 0.1	13.8 ± 0.2	17.7 ± 0.3	12.7 ± 0.2	14.0 ± 0.2	18.4 ± 0.4
CV	0.2	0.2	0.25	0.20	0.25	0.22	0.14	0.29
H (min–max, %)	48.3–99.9	66.2–99.9	69.3–99.9	53.2–87.7	31.9–77.3	42.8–92.3	54.2–96.1	36.2–90
Mean H ± SE (%)	80.7 ± 1	85.9 ± 0.5	86.6 ± 0.5	74.9 ± 0.5	55.7 ± 0.7	72.6 ± 0.7	73.2 ± 0.7	64.8 ± 1.1
CV	0.2	0.11	0.09	0.11	0.21	0.17	0.09	0.23

CV – coefficient of variation.

ence in 2 × 2 contingency tables. Only combinations that gave expected numbers ≥ 5 in all cells were included in the analysis. Independence among tests was enabled by omission of the least frequent arrangement of each of five chromosomes, except for J chromosome where only two gene arrangements were recorded. LD was calculated only for haplotype I. The results for haplotype II have the same value with the opposite sign. Bonferroni correction was applied.

Microsatellite variability

Eleven polymorphic microsatellite loci were amplified with primers designed by Pascual et al. (2000, 2001). Two microsatellite loci were analysed per chromosomes A, J, U and E (A: dsub05, dsub19; U: dsub03, dsub15; E: dsub13, dsub20; J: dsub18, dsub27). On the O chromosome, the longest one, three microsatellite loci were genotyped (dsub01, dsub02, dsub04) (Santos et al., 2010). Microsatellite loci were amplified using PCR in four multiplex reactions: 1 (dsub27, dsub20, dsub04), 2 (dsub01, dsub18, dsub05), 3 (dsub02, dsub13, dsub19) and 4 (dsub03, dsub15). Four different fluorescent dyes were used to end-label one primer of each primer pair (FAM – dsub01, dsub04, dsub19; NED – dsub02, dsub13, dsub18; PET – dsub05, dsub15, dsub20 and VIC – dsub03, dsub27). The PCR conditions were similar to those of Pascual et al. (2000): 5 min at 95°C followed by 30 cycles of denaturing at 95°C for 1 min, annealing at 57°C for 30 s and extension at 72°C for 30 s. The exception was that a final elongation of 30 min at 60°C was included and 2 μL (50 μg/μL) of DNA were added to the total volume (20 μL) of the PCR mix. PCR products of amplifications of reactions 1 and 2 were mixed, as the products of the reactions 3 and 4, because coupled products labelled with the same dye did not overlap in the sizes of fragments.

Fragment length was determined using GeneScan LIZ 600 size standard and GeneMapper software on an automated sequencer ABI Prism 3130 (Thermo Fisher Scientific).

Levels of polymorphism for each locus, each population and all loci and populations were estimated using Arlequin v.3.5 software (Excoffier & Lischer, 2010) by calculating the mean number of alleles – allelic richness (A), mean range in allele size (R), expected heterozygosity (H_E) and observed heterozygosity (H_O). Genetic variability between sampled localities was analysed by the analysis of molecular variance (AMOVA) with the same

software. We used the F_{ST} distance method (Weir & Cockerham, 1984; Excoffier et al., 1992; Weir, 1996).

The possible occurrence of a recent reduction in population size was tested separately for all samples using Bottleneck software (v.1.2.02) (Cornuet & Luikart, 1996). Wilcoxon signed rank test was used, and two phase mutation model was assumed, since Pascual et al. (2001) demonstrate its accuracy on a similar set of microsatellite loci.

Populations' genetic structure and environmental factors

To test whether genetic differences between populations could be attributed to distance between populations, the Mantel test (Mantel, 1967) was applied in PAST software (Hammer et al., 2001) using log-transformed distances and F_{ST} values for inversion polymorphism.

Genetic differences based on microsatellite and inversions data were compared using a principal component analysis (PCA) on the allelic frequencies of samples. In order to estimate general pattern within them, as well as within the environmental dataset, correlation loadings between original variables and principal axes (eigenvectors) were obtained.

In order to examine whether patterns of genetic variation correlate with patterns of environmental variation, we performed correlation and regression analyses. The joint effects of environmental variables on chromosomal gene arrangement frequencies was examined with PCs, obtained by principal component analysis on environmental data and individual chromosomal arrangements. The association of individual environmental variables with joint chromosomal gene arrangements was determined using PCs, obtained by PCA of inversion frequencies and individual environmental variables.

In order to identify environmental factors that might be associated with the genetic structure of *D. subobscura* populations we used the hierarchical Bayesian approach of Foll & Gaggiotti (2006) implemented in GESTE v. 2.0. Specifically, GESTE estimates F_{ST} values for each local population and relates them to environmental factors using a generalized linear model. As this method requires genetic data from codominant markers (e.g. allozymes, microsatellites, or SNPs) and environmental data specific to each local population, we analysed only microsatellites and inversion data. We performed 10 pilot runs of 1000 iterations and default parameters to obtain the parameters of the proposed distributions used by the reversible jump Markov chain Monte

Table 3. The results of AMOVA for chromosomal gene arrangements, RFLP of *ND5* gene and microsatellites.

Genetic marker	d.f.	Source of variation	Sum of squares	Variance components	% of variation	F_{ST}	*P*-value
Inversion polymorphism	7 740	ap wp	24.94 840.17	0.026 1.135	2.240 97.76	0.022	< 0.0001*
RFLP of *ND5*	7 344	ap wp	3.14 84.58	0.005 0.246	1.840 98.16	0.018	0.076[ns]
Microsatellite loci	7 678	ap wp	34.65 2602.43	0.013 3.838	0.34 99.66	0.003	0.087[ns]

n.s. – non-significant; *P*-value < 0.001; ap – among population; wp – within population.

Table 4. Gene arrangement frequencies for all chromosomes in all eight populations.

Population	B	BR	VB	PR	H	KH	BZ	NB
N	52	52	49	39	44	51	49	38
A_{ST}	0.902	0.655	0.640	0.744	0.717	0.698	0.720	0.725
A_1	0.039	0.255	0.220	0.163	0.239	0.245	0.240	0.175
A_2	0.059	0.091	0.140	0.093	0.043	0.057	0.040	0.100
J_{ST}	0.363	0.282	0.300	0.372	0.293	0.189	0.230	0.425
J_1	0.637	0.718	0.700	0.628	0.707	0.811	0.770	0.575
U_{ST}	0.137	0.091	0.040	0.116	0.120	0.047	0.070	0.188
U_1	0.000	0.000	0.000	0.000	0.022	0.009	0.020	0.000
U_{1+2}	0.735	0.655	0.790	0.663	0.663	0.679	0.720	0.700
U_{1+2+6}	0.127	0.255	0.170	0.221	0.196	0.264	0.190	0.113
E_{ST}	0.422	0.227	0.270	0.349	0.435	0.226	0.240	0.500
E_8	0.225	0.218	0.210	0.233	0.370	0.311	0.180	0.300
E_{1+2+9}	0.324	0.509	0.500	0.384	0.163	0.425	0.570	0.163
$E_{1+2+9+12}$	0.029	0.045	0.020	0.035	0.033	0.038	0.010	0.038
O_{ST}	0.343	0.327	0.150	0.430	0.391	0.264	0.230	0.463
O_6	0.000	0.000	0.000	0.000	0.000	0.000	0.010	0.000
O_{3+4}	0.441	0.409	0.550	0.442	0.359	0.519	0.490	0.388
O_{3+4+1}	0.186	0.227	0.260	0.128	0.207	0.179	0.230	0.150
O_{3+4+2}	0.029	0.036	0.040	0.000	0.043	0.038	0.040	0.000
HZ	0.598	0.623	0.550	0.622	0.592	0.623	0.600	0.581

N – number of analysed chromosomes; HZ – degree of heterozygosity.

Carlo method implemented in GESTE. We further applied an additional burn-in of 50,000 iterations and a thinning interval of 20. All estimates were derived from a sample size of 10,000.

RESULTS

Inversion polymorphism

We evaluated the level of genetic variability using inversion polymorphisms of all five acrocentric chromosomes (A, J, U, E, and O). A total of 18 chromosomal arrangements were detected in the whole data set, among them 16 were found in all populations. U_1 chromosomal arrangement was found in three populations only (H, KH and BZ), while arrangement O_6 was found only in the BZ population with a very low frequency (0.01). Gene arrangement frequencies are shown in Table 4. Heterozygosity of all samples was similar.

Z-tests (Table S1) clearly showed significant differences in chromosomal arrangement frequencies between pairs of populations, for all five chromosomes, but most differences were for the E chromosome, especially in the E_{1+2+9} arrangement. Large numbers of differences were obtained for the E_{ST} and O_{ST} arrangements.

The F_{ST} analysis (Table 5) showed that the B, H, NB and PR populations are significantly differentiated from the other four: BR, KH, VB and BZ.

MtDNA haplotype frequency distributions in the populations sampled

Haplotype II was more frequent in populations H, PR, KH, VB, BZ, while haplotype I was more frequent in B, NB and BR. The global test of genetic differentiation was not significant (F_{ST} = 0.0184; P = 0.0762) (Table 3), with the greatest source of variation within putative populations (98.16%). Most pairwise comparisons were not statistically significant, except between B and H ($P < 0.05$), B and BZ ($P < 0.01$) and NB and H ($P < 0.01$) localities (Table 5).

Linkage disequilibrium between mtDNA and inversion polymorphism

The power of the LD analysis of separate populations was low (Table S2). Many arrangements did not fulfil the criteria for testing due to their low frequency within samples. Two populations (H and VB) showed weak LD for the same gene arrangement (O_{3+4}) and haplotype I. For population H, the D' value was 0.319 ($P < 0.01$) whereas for VB it was 0.236 ($P < 0.05$). Considering the whole sample, weak LD was recorded for the O_{ST} arrangement and hap-

Table 5. Population pairwise F_{ST}-values based on chromosomal arrangement, haplotype and microsatellite frequencies. After Bonferroni correction only *** labelled values remained significant.

Population	Marker	B	H	PR	NB	BR	KH	VB
H	M.S.	0.0015						
	ND5	**0.1256***						
	I.P.	0.0064						
PR	M.S.	0.0028	0.0076					
	ND5	0.0147	0.0141					
	I.P.	0.0013	0.0115					
NB	M.S.	0.0055	0.0051	0.0070				
	ND5	−0.0247	**0.1060****	0.0023				
	I.P.	0.0014	0.0065	0.0023				
BR	M.S.	0.0043	0.0014	0.0061	0.0033			
	ND5	−0.0097	0.0614	−0.0151	−0.0171			
	I.P.	**0.0165****	**0.0265****	0.0034	**0.0362****			
KH	M.S.	0.0008	−0.0014	0.0043	0.0010	−0.0009		
	ND5	0.0268	0.0086	−0.0249	0.0134	−0.0073		
	I.P.	**0.0297****	**0.0339****	**0.0234****	**0.0616****	0.0021		
VB	M.S.	**0.0076****	0.0038	0.0034	0.0017	−0.0004	0.0006	
	ND5	0.0703	−0.0143	−0.0128	0.0534	0.0204	−0.0141	
	I.P.	**0.0321****	**0.0564****	**0.0320****	**0.0652****	**0.0106****	0.0066	
BZ	M.S.	**0.0095****	**0.0076***	0.0024	0.0041	0.0040	0.0021	0.0033
	ND5	**0.0808****	−0.0176	−0.0085	0.0633	0.0278	−0.0107	−0.0216
	I.P.	**0.0306****	**0.0544****	**0.0233****	**0.0631****	0.0005	0.0040	0.0035

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Pop – population; M.S. – microsatellites; I.P. – inversion polymorphism.
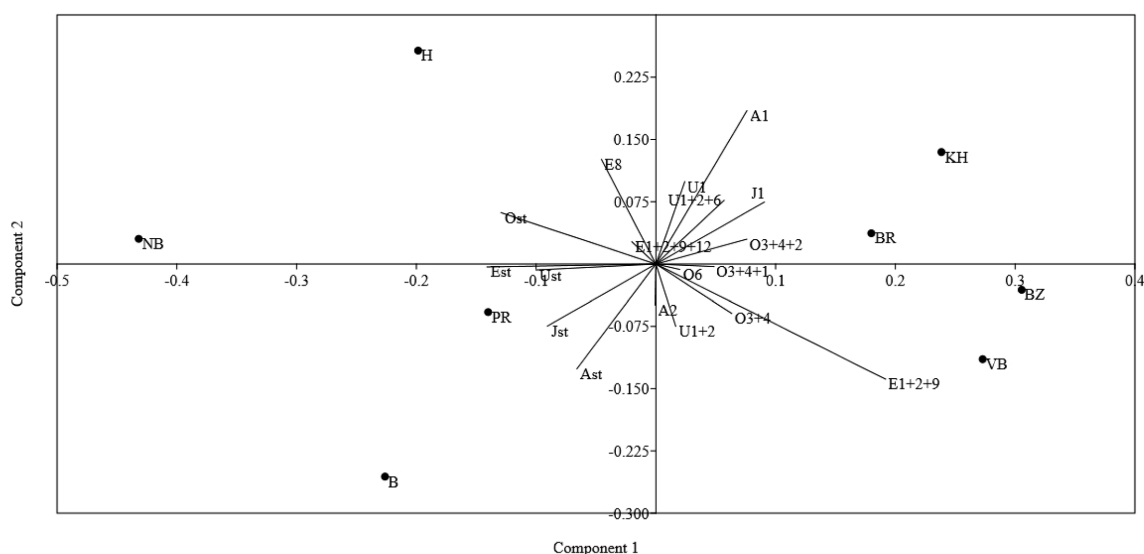
Fig. 2. Biplot of correlation coefficients (loading factors) of chromosome arrangements frequencies with PCA axes 1 and 2.

lotype II (Table S3). This is the consequence of same sign of D' value in seven out of eight populations. Other gene arrangements did not show a similar trend across populations. After Bonferroni correction, none of the observed D' value was significantly different from zero.

Microsatellite variability

Standard measures of genetic diversity of microsatellites are presented in the Table S4. The number of alleles varied from 7 (locus dsub13, population H) to 25 (locus dsub18, population BR). The mean number of alleles was similar between populations. Expected heterozygosity's were high and very similar in all populations. In all populations, the average observed heterozygosity was lower than the average expected heterozygosity.

The AMOVA showed that the majority of variation is present within populations (99.66%) (F_{ST} = 0.0034; P = 0.087) (Table 3). Most pairwise comparisons were not statistically significant, except between B and VB ($P < 0.001$), B and BZ ($P < 0.01$) and H and BZ ($P < 0.05$) localities (Table 5).

The Bottleneck analysis didn't reveal a significant heterozygote excess in populations. In terms of the TPM model there were no significant results regarding recent genetic bottleneck effects on the populations (all $P > 0.05$ for Wilcoxon test).

Populations' genetic structure and environmental factors

The Mantel test revealed that the correlation coefficient (bootstrapped ordinary least squares regression analysis with 95% confidence intervals) was extremely low (R^2 = 0.0223, P = 0.4535), implying that around 97% of the observed genetic variation was not associated with geographic distance.

PCA was done using the frequencies of microsatellite loci, inversion arrangements and environmental data. PC analysis on inversion chromosomal arrangements data revealed that PC_1 explains 57.012% of the variation of inversions in all populations, while PC_2 and PC_3 explain

17.448% and 11.263%, respectively. PC_1 had high positive loading for E_{1+2+9} (0.53), moderate for J_1, A_1, O_{3+4+2} (0.25; 0.21; 0.21 respectively), high negative loadings for standard arrangements of chromosomes E and O (–0.39; –0.36 respectively) and moderate for U and J (–0.28; –0.25 respectively). So, the loading coefficients of PC_1 reveal a weighted contrast between all standard chromosomal gene arrangements (with negative loadings), against the majority complex and simple arrangements (with positive loadings). PC_2 had higher positive loadings for A_1 and E_8 (0.51; 0.35 respectively) and high negative loadings for A_{ST} and E_{1+2+9} (–0.39; –0.35 respectively) (Fig. 2).

PC analysis of microsatellite data revealed that PC_1 explains 18.203% of the variation of microsatellites in all populations, while PC_2 and PC_3 explain 16.008% and 14.884%, respectively. The first three principal components explained only 49.095% of total variance (unlike variance of inversion arrangement data: 85.723%). As the variation recorded by this analysis was minor any further microsatellite analyses were not performed.

The third PCA was performed using environmental variables: the mean temperature, humidity and altitude. The first principal and second components explained 76.485% and 18.72% of the total variation, respectively (95.205% together). Altitude and temperature are mostly associated with PC_1 with positive (0.63) and negative loading (–0.58) respectively, but humidity (with positive loading 0.82) was mostly associated with PC_2.

In order to determine the environmental factors that might be associated with the genetic structure of *D. subobscura* populations, we analysed using linear regression the associations of environmental variables with inversion frequencies. When individual chromosome arrangements were considered, the frequencies of E_{ST}, E_{1+2+9} and $E_{1+2+9+12}$ were significantly correlated with only the second principal component of environmental variables. The frequency of E_{1+2+9} (r = –0.81, P = 0.015) was negatively correlated, unlike the $E_{1+2+9+12}$ (r = 0.78, P = 0.021) and E_{ST} (r = 0.73, P = 0.041) chromosome arrangements.

When environmental variables were considered, only temperature was significantly correlated with inversion first principal component ($R^2 = 0.56$, $P = 0.041$). Altitude and humidity were not correlated with inversion PCs.

Regression approaches employed in GESTE revealed that runs with just a single environmental factor did not produce higher probability models when the factor was included in the model compared to when it was excluded. The model that included mean temperature and the constant had the second highest posterior probability (0.166), while the models containing mean humidity and the constant had a much lower posterior probability (0.091), as also did that with altitude and the constant (0.084). The importance of temperature was supported when looking at the data fit with just the factors alone, because temperature had a posterior probability of 0.291 (humidity and altitude had 0.189 and 0.170, respectively). We could not analyse the interaction of factors because they were significantly correlated.

DISCUSSION

The present results on population differentiation obtained using three genetic markers differ markedly. They provide evidence that local adaptations are associated with chromosomal inversions and mito-nuclear epistasis at a very fine spatial scale even in populations of *D. subobscura* with high dispersal ability (Begon et al., 1980; Ayala et al., 1989) and high effective population size (Pascual et al., 2001; Kurbalija Novicic et al., 2011, 2013; Stamenkovic-Radak et al., 2012).

The adaptively neutral nuclear microsatellites were highly variable in all populations. Low share of among population variation, obtained by AMOVA and low values of the F_{ST} indices of pairwise comparisons indicate an absence of neutral genetic differentiation. More importantly, none of the populations have experienced recent bottleneck, which could result in nonadaptive differences between populations.

On the other hand, both AMOVA and pairwise comparisons revealed a higher differentiation based on mitochondrial *ND5* gene than that based on microsatellites. Frequencies of haplotypes were in accordance with previously published data (Christie et al., 2010; Jelic et al., 2012a, b). Abundant literature on *D. subobscura* indicate that both adaptively neutral and selective processes have shaped its mtDNA variability (Castro et al., 2003, 2010; Christie et al., 2004, 2010, 2011; Arnquist et al., 2016). Generally, the previously reported repeatable seasonal dynamics of frequencies (Christie et al., 2010) indicate that mtDNA has been subjected to natural selection. The observed mitochondrial differentiation can be at least partly attributable to stochastic processes, since selection acting on the mt genome is not efficient compared to that on nuclear genes. In this species there are population decays induced by unfavourable conditions during cold winters and dry summers, which are followed by expansions (Castro et al., 2010; Christie et al., 2010) that leave traces in mtDNA variability, in excess of singletons.

Some of the differences in mtDNA frequencies across populations may be at least partly adaptive, especially in light of natural selection acting on joint allelic combinations of the nuclear and mitochondrial genomes. Fitness differences recorded in a laboratory of bearers of different haplotypes of *D. subobscura* indicate a scenario of epistatic interaction between nuclear and mitochondrial genomes (Christie et al., 2011). Chromosomal inversions are a particularly interesting marker for assessing epistatic interactions in natural populations since the coadaptation hypothesis predicts presence of different alleles in different gene arrangements (Dobzhansky, 1948; Hoffmann et al., 2004). This approach was used to determine the LD between chromosomal and mitochondrial variability in several natural populations of *D. subobscura* across its range (Oliver et al., 2002; Jelic et al., 2012a, b). Inconsistency in LD results of these studies may indicate temporally or spatially variable epistasis, which is supported by experimentally proven environmentally specific epistasis in insects (Dowling et al., 2007; Arnqvist et al., 2010; Mossman et al., 2016; Rand et al., 2018). Our results are in agreement with this notion since we recorded weak LD between O_{3+4} gene arrangement and haplotype I, but only in some of the populations. We show that on a small spatial scale, where there is high gene flow, only subtle mito-nuclear LD occurs.

Gregorius & Ross (1984) propose that negative frequency dependant selection (NFDS) acts to maintain the variation in mtDNA. This was experimentally shown in the seed beetle *Callosobruchus maculates* (Kazancioglu & Arnqvist, 2014) and the two main haplotypes of *D. subobscura* (Oliver et al., 2005; Arnqvist et al., 2016). Contrary to the findings of Arnqvist et al. (2016), García-Martinez et al. (1998) did not record the maintenance of the two haplotypes but favouring and fixation of haplotype II. One possible reason for this discrepancy could be the different environmental conditions in these studies (Arnqvist et al., 2016). So NFDS can act differently depending on the environment. If it acts strongly it will lead to similar frequencies of the two haplotypes, and in its absence there may be greater shifts in the haplotype frequencies.

A great body of evidence supports the adaptive significance of chromosomal variability in *D. subobsura*. The most illustrative proof comes from the accidental introduction of this species from the Palearctic to South and North Americas. Signs of the correlation coefficients between chromosomal arrangement frequencies and latitude are highly coincident in both native and colonized regions (Prevosti, 1988; Balanya et al., 2003). Among the markers analysed in this study inversion polymorphism revealed the highest genetic differentiation between our closely located populations. Share of between population differentiation is also low for inversions, but nevertheless it is an order of magnitude higher than for neutral microsatellites, and this share is significantly different from zero. Comparisons of differentiation of these two nuclear markers, together with the absence of population bottlenecks indicate that differences between populations in inversion polymorphism are

adaptive. In addition, most of these adaptive differences were recorded for chromosome E.

Although we detected signs of local adaptation based on chromosomal polymorphism, it is still an open question whether an abiotic and/or biotic factor is mostly responsible for the divergence. It is important to consider the limitations of the approaches used in this study. As we only recorded environmental data over a short interval prior and during sampling the flies we cannot have defined the environmental factors that act throughout the year. The sampling locations were very close, and world climatic data were not applicable in our case. But it is reasonable to assume that the relations of shortly assessed parameters between populations in some respect mirror the long standing ones. In other words, a warm habitat assessed in this way was more likely to be warmer throughout the year due to constant exposure and altitude. Our results indicate it was mostly differences in temperature that were associated with the variation in gene arrangement frequencies between populations. This is particularly the case for the complex arrangement E_{1+2+9}, which is on a chromosome that differs most between populations. The effect of temperature on shaping inversion polymorphism in *D. subobscura* has been previously addressed experimentally, more specifically the thermal preference and heat tolerance of individuals of known genetic backgrounds (Dolgova et al., 2010; Rego et al., 2010). The results indicate that variation in these two traits is not genetically correlated and is associated with different parts of the genome. Dolgova et al. (2010) show that individuals with the warm adapted complex arrangements of O chromosome have a higher thermal preference. On the other hand, there are no associations between O chromosomal inversions and heat tolerance. The study of Rego et al. (2010) is not restricted to particular chromosomes. Generally, the carriers of cold-adapted gene arrangements choose low temperatures and are less tolerant to heat stress. However, only variation in chromosome A, and to lower extent in chromosome O, are associated with the thermal preference of *D. subobscura*. On the other hand, only variation in chromosome E is significantly associated with tolerance of high temperatures. The association between heat tolerance and chromosome E, however, are particularly important. They show that individuals collected in this study not only chose habitats based on their thermal optimum, but more importantly they were adapted to tolerate heat stress, which varies between local populations. Thermal tolerance is of immense importance for survival, since temperature is quite variable in moderate climates (Rego et al., 2010). The importance of temperature, especially T_{min} and $T_{max,}$ was confirmed in this species when inversion polymorphism was analysed in one population in five consecutive years (Galludo et al., 2018).

Our findings indicate that the longstanding view that local adaptation only occurs at large geographical scales due to selection-migration balances should be reviewed. We propose that locally adapted alleles within chromosomal inversions forming coadaptive gene complexes, together with joint selection acting on mitochondrial and nuclear genomes, are responsible for the presence of local adaptations to specific microhabitats in *D. subobscura*, despite high gene flow. This hypothesis is supported by the significant outbreeding depression reported by Kurbalija et al. (2010) in inter-population hybrids from geographically close but environmentally distinct microhabitats. The occurrence of significant outbreeding depression in the F_1 generation could be explained by factors such as underdominance, epistatic interactions or disruption of local adaptations (Edmands, 2007; Escobar et al., 2008) as in the cited study.

This study did not delineate in detail the action of environmental effects on local adaptation. However, this does not compromise our findings of local adaptive differentiation that is apparent from chromosomal variation across populations and possibly the variable presence of LD across populations, both of which are subordinate to the complex synergy of locally present ecological environment. We demonstrate local adaptation on a small spatial scale in a species with high dispersal ability, so the focus of future studies should be shifted from questioning its existence to factors and processes that maintain it.

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Table S1. Differences in inversion arrangement frequencies obtained using *Z* statistics.

	A_{ST}	A_1	A_2	J_{ST}	J_1	U_{ST}	U_1	U_{1+2}	U_{1+2+6}	E_{ST}	E_8	E_{1+2+9}	$E_{1+2+9+12}$	O_{ST}	O_6	O_{3+4}	O_{3+4+1}	O_{3+4+2}
NB/H				*	*				*									
NB/B	**	**										***		*				*
NB/VB				*	*	***		*		***	*	***		***		**	*	*
NB/BZ				***	***	***			*	***	**	***		***				*
NB/BR				**	**	**			***	***		***		**				*
NB/KH				***	***	***			***	***		***		***		*		*
NB/PR									**	**		***						
H/B	***	***					*				**	***						
H/VB			*			**	*	**		***	***	***		***		***		
H/BZ										***	***	***		***				**
H/BR							**			***	***	***						
H/KH				*	*	**				***		***		**		**		
B/VB	***	***				***				**		***		***		*		
B/BZ	***	***	**	**	**	*	*			***		***		*				
B/BR	***	***							***	***		***						
B/KH	***	***		***	***	**			***	***		*						
VB/BZ			*				*							*				
VB/BR						*		**						***		**		
VB/KH				**	**			*	*		*			**		*		
BZ/BR							*						*	*				
BZ/KH											**	**						
BR/KH				*	*											*		
PR/H											**	***					*	**
PR/B	**	**							*									*
PR/VB						**		**				*		***		*	**	**
PR/BZ				**	**					*		***		***			*	**
PR/BR										**	*			*			*	*
PR/KH				***	***	*				**				***				**

*P < 0.05, **P < 0.01, ***P < 0.001.

Table S2. LD analysis for each population.

	H				B				PR				NB		
	D	D'	P		D	D'	P		D	D'	P		D	D'	P
A_{st}	0.0058	0.0513	–	A_{st}	0.0232	0.328	–	A_{st}	0.0048	0.044	–	A_{st}	–0.0082	–0.0667	–
A_1	–0.0139	–0.1461	–	A_1	–0.0045	–0.16	–	A_1	–0.0273	–0.4423	–	A_1	0.0163	0.222	–
A_2	0.008	0.2292	–	A_2	–0.0187	–0.44	–	$\underline{A_2}$	0.0226	0.3958	–	A_2	–0.0082	–0.125	–
J_{st}	–0.0234	–0.2238	0.4312	J_{st}	0.0017	0.0118	1	J_{st}	0.0369	0.1761	0.285	J_{st}	–0.0163	–0.0667	0.631
J_1	0.0234	0.2238	0.4312	J_1	–0.0017	–0.0118	1	J_1	–0.0369	–0.1761	0.285	J_1	0.0163	0.0667	0.631
U_{st}	0.0288	0.2993	0.1884	U_{st}	–0.0232	–0.328	–	U_{st}	0.038	1	–	U_{st}	–0.0204	–0.1923	0.537
U_1	–0.0095	–1.0000	–	U_1	–	–	–	U_1	–	–	–	U_1	–	–	–
U_{1+2}	–0.0259	–0.1287	0.2802	U_{1+2}	–0.0227	–0.2139	–	U_{1+2}	–0.0868	–0.4563	0.067	U_{1+2}	0.0061	0.0375	0.537
U_{1+2+6}	0.0066	0.0750	–	U_{1+2+6}	0.0459	0.7941	–	U_{1+2+6}	0.0487	0.3203	0.067	U_{1+2+6}	0.0143	0.3333	–
E_{st}	–0.0161	–0.1167	0.6181	E_{st}	–0.0099	–0.042	0.821	E_{st}	–0.0244	–0.1502	0.422	E_{st}	–0.0388	–0.1979	0.457
E_8	0.0292	0.1338	0.3163	E_8	–0.0204	–0.16	0.421	E_8	0.0392	0.275	0.236	E_8	–0.0041	–0.0454	1
E_{1+2+9}	–0.0124	–0.1868	–	E_{1+2+9}	0.0278	0.2059	0.342	E_{1+2+9}	–0.0071	–0.044	0.789	E_{1+2+9}	0.0245	0.2222	0.283
$E_{1+2+9+12}$	–0.0007	–0.0513	–	$E_{1+2+9+12}$	0.0025	0.1765	–	$E_{1+2+9+12}$	–0.0077	–1	–	$E_{1+2+9+12}$	0.0184	1	–
O_{st}	–0.0383	–0.2885	0.3048	O_{st}	–0.0317	–0.16	0.347	O_{st}	–0.0053	–0.0301	1	O_{st}	–0.0531	–0.2222	0.074
O_6	–	–	–	O_6	–	–	–	O_6	–	–	–	O_6	–	–	–
O_{3+4}	0.0698	0.3194	**0.009****	O_{3+4}	–0.0051	–0.0221	1	O_{3+4}	–0.0113	–0.0562	1	O_{3+4}	0.049	0.2759	0.074
O_{3+4+1}	–0.0442	–0.6205	–	O_{3+4+1}	0.0224	0.2734	0.28	O_{3+4+1}	0.0166	0.1944	–	O_{3+4+1}	0.0041	0.0667	–
O_{3+4+2}	0.0128	0.4861	–	O_{3+4+2}	0.0145	1	–	O_{3+4+2}	–	–	–	O_{3+4+2}	–	–	–

	BR				KH				VB				BZ		
	D	D'	P		D	D'	P		D	D'	P		D	D'	P
A_{st}	–0.0523	–0.3478	0.7381	A_{st}	–0.0248	–0.1538	1	A_{st}	0.019	0.1296	–	A_{st}	–0.0136	–0.0739	1
A_1	0.0081	0.0761	0.7381	A_1	0	0	1	A_1	–0.0045	–0.0505	–	A_1	0.0086	0.0546	1
A_2	0.0442	1	–	A_2	0.0248	1	–	A_2	–0.0145	–0.254	–	A_2	0.005	0.1897	–
J_{st}	0.0159	0.1289	0.5104	J_{st}	–0.0041	–0.0571	1	J_{st}	–0.0258	–0.2879	0.317	J_{st}	0.0167	0.116	0.461
J_1	–0.0159	–0.1289	0.5104	J_1	0.0041	0.0571	1	J_1	0.0258	0.2879	0.317	J_1	–0.0167	–0.116	0.461
U_{st}	0.0148	0.3345	–	U_{st}	–0.0031	–0.12	–	U_{st}	–0.0057	–0.3472	–	U_{st}	0.0075	0.1897	–
U_1	–	–	–	U_1	–0.0052	–1	–	U_1	–	–	–	U_1	0.0066	1	–
U_{1+2}	–0.0165	–0.1011	0.8217	U_{1+2}	0.031	0.2143	0.353	U_{1+2}	0.0177	0.2167	0.58	U_{1+2}	–0.0045	–0.0276	1
U_{1+2+6}	0.0017	0.0145	0.8217	U_{1+2+6}	–0.0227	–0.2	0.332	U_{1+2+6}	–0.012	–0.184	0.58	U_{1+2+6}	–0.0095	–0.1296	0.79
E_{st}	–0.0169	–0.1257	0.4896	E_{st}	0.0248	0.2667	0.249	E_{st}	–0.0511	–0.5459	–	E_{st}	0.0061	0.0423	0.808
E_8	–0.0017	–0.0134	1	E_8	–0.0124	–0.0774	1	E_8	0.0143	0.1086	0.603	E_8	–0.0201	–0.2747	0.42
E_{1+2+9}	0.0161	0.0761	0.5426	E_{1+2+9}	–0.032	–0.1676	0.383	E_{1+2+9}	0.0344	0.1876	0.197	E_{1+2+9}	0.0181	0.1084	0.668
$E_{1+2+9+12}$	0.0025	0.113	–	$E_{1+2+9+12}$	0.0196	0.6333	–	$E_{1+2+9+12}$	0.0025	0.1896	–	$E_{1+2+9+12}$	–0.0041	–1	–
O_{st}	–0.0359	–0.1964	0.2102	O_{st}	–0.0165	–0.1391	0.474	O_{st}	–0.0317	–0.5983	–	O_{st}	0.0167	0.116	0.617
O_6	–	–	–	O_6	–	–	–	O_6	–	–	–	O_6	–0.0041	–1	–
O_{3+4}	–0.005	–0.0226	1	O_{3+4}	0.0403	0.1814	0.191	O_{3+4}	0.0394	0.2358	**0.049***	O_{3+4}	–0.045	–0.2457	0.088
O_{3+4+1}	0.0233	0.2287	0.2345	O_{3+4+1}	–0.0196	–0.2235	0.418	O_{3+4+1}	–0.0233	–0.2384	0.462	O_{3+4+1}	0.038	0.2633	0.132
O_{3+4+2}	0.0177	1	–	O_{3+4+2}	–0.0041	–0.2667	–	O_{3+4+2}	0.0156	0.5948	–	O_{3+4+2}	–0.0057	–0.3472	–

*$P < 0.05$, **$P < 0.01$.

Table S3. Whole sample LD analysis.

	D	D'	Two tailed P value
A_{ST}	–0.0036	–0.0231	0.7840
A_1	–0.0053	–0.0550	0.7840
A_2	0.0088	0.1961	–
J_{ST}	0.0035	0.0227	0.7309
J_1	–0.0035	–0.0227	0.7309
U_{ST}	0.0054	0.1068	0.3567
U_1	–0.0013	–0.4645	–
U_{1+2}	–0.0105	–0.067	0.1981
U_{1+2+6}	0.0064	0.0621	0.4316
E_{ST}	–0.0107	–0.0732	0.3114
E_8	–0.0016	–0.0134	1.0000
E_{1+2+9}	0.0073	0.0341	0.3356
$E_{1+2+9+12}$	0.0049	0.2660	–
O_{ST}	–0.0200	–0.1411	**0.0278***
O_6	–0.0007	–1.0000	/
O_{3+4}	0.0129	0.0529	0.2114
O_{3+4+1}	0.0016	0.0142	0.9235
O_{3+4+2}	0.0063	0.3748	0.0761

*$P < 0.05$.

Table S4. Parameters of microsatellite variability.

Population	A	R	H_O	H_E
B	13.64	19.36	0.706	0.821
H	12.91	18.64	0.673	0.823
PR	14.18	17.73	0.732	0.835
NB	13.73	19.27	0.688	0.852
BR	15.91	25.91	0.732	0.846
KH	14.45	20.64	0.744	0.849
VB	14.45	20.64	0.721	0.853
BZ	14.36	20.36	0.694	0.847

A – mean allelic richness; R – mean allelic range; H_O – observed heterozygosity; H_E – expected heterozygosity.