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Balkan glacial history and modern *Drosophila subobscura* population genetics

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ABSTRACT

Background: The Balkan Peninsula was one of three main refugia for many European species during the last glaciation. During that period, glacial–interglacial climate oscillations probably shaped the habitat characteristics and influenced the population genetic structures of the Balkan species we see today, including *Drosophila subobscura*.

Hypothesis: Some central Balkan gorges were refugia for *D. subobscura*. We can detect the effects of these refugia by identifying specific patterns of genetic variability using different genetic markers. We expect conspecific populations to be geographically structured and to differ in particular parameters of population genetic structure.

Methods: We analyse the genetic structure of six *D. subobscura* populations that occupy geographically and ecologically different habitats from the central part of the Balkans using inversion polymorphism, microsatellites, and mtDNA markers.

Results: The variability generally fits the European population distribution, with specific gene pool structure in some local populations. Populations from gorges are distinctive in particular rare gene arrangements, mtDNA haplotype diversity providing negative Tajima *D*-values, whereas the analysed microsatellite loci did not show significant departures from neutrality.

Conclusions: The degree and pattern of the differences leaves open the question of the refugial existence of *D. subobscura* in the central Balkans. The inversion polymorphism data show that some endemic arrangements are present with high frequency. But the molecular markers do not fully support the refugial hypothesis. The adaptive divergence we see among the populations indicates that local adaptations at the molecular level can occur despite high gene flow and large effective population sizes.

Keywords: adaptive evolution, glacial refugia, inversion polymorphism, microsatellites, mtDNA.

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INTRODUCTION

The reconstruction of paleo-vegetation maps indicates three main glacial refugia of deciduous temperate forest located in the southern European peninsulas of Iberia, Italy, and the Balkans where temperate species that are today widespread across Europe must have survived in small and climatically favourable areas (Hewitt, 2000). Due to severe glacial–interglacial climatic oscillations during the last 2 million years, temperate flora and fauna are expected to have gone through many contractions and range expansions, which have left signatures in the geographical distribution and genetic diversity of extant populations.

The importance of refugia is increasingly being recognized (Noss, 2001; Barnosky, 2008; Bennett and Provan, 2008; Rull, 2009; Stewart *et al.*, 2010). The focus of many contemporary studies in conservation biology is the impact of pre-history on genetic structure, based on present population genetic information, so that we can predict the effects of present climate and environmental changes on the genetic future of populations of different species of mammals, fish, and birds (Valdiosera *et al.*, 2007; Maggs *et al.*, 2008). Refugial populations that evolved in allopatry are expected to have accumulated independent genetic differences that may be used as genetic markers to trace expansion routes. It is also expected that genetic variability is higher in refugial populations, since the latter are characteristically formed by a subset of the original gene pool.

Drosophila subobscura has a broad Palearctic distribution and occurs on some Atlantic islands, and in the late 1970s colonized South and North America where it spread rapidly and successfully. Many studies have focused on population genetic structure screening of different natural populations of *D. subobscura*, based on inversion polymorphism (Prevosti, 1974; Prevosti *et al.*, 1988; Krimbas, 1993; Andjekovic *et al.*, 2007; Kenig *et al.*, 2010), allozymes (Marinkovic *et al.*, 1978; Pinto *et al.*, 1997; Castro *et al.*, 1999), mtDNA (Afonso *et al.*, 1990; Latorre *et al.*, 1986, 1992; García-Martínez *et al.*, 1998; Castro *et al.*, 1999) and, recently, nuclear molecular analysis (Pascual *et al.*, 2000, 2001; Kurbalija Novicic *et al.*, 2011). The results of these studies suggest that different markers show different amounts of geographic variation.

Here, we focus on the pattern of genetic diversity among six *D. subobscura* populations of the central Balkans. Among these, two populations are from gorges and one from a canyon, all recognized as refugia in terms of their floristic and faunistic data, and their specific natural habitats are probably shaped by glacial–interglacial climate oscillations. The genetic variability of these populations was analysed by integrating data from multiple genetic markers (chromosomal inversions, mtDNA, and microsatellite variability) to reveal the present genetic structure and possibly deduce the historical processes that shaped it.

MATERIALS AND METHODS

Drosophila subobscura flies were sampled at six localities in Serbia: Goc Mountain (G), Botanical Gardens (BG), Deliblato Sands (DS), Derventa River Gorge (DRG), Sicevo Gorge (SG), and Lazar River Canyon (LRC). The Goc Mountain sample (43°N, 20°E) was from beechwood (*Abieto-fagetum*) and oakwood (*Fraxineto-quercetum*) forests. The Botanical Gardens (Arboretum) locality (44°49′00″N, 20°28′24″E) in central urban Belgrade has a microclimate affected by marked anthropogenic activity. Deliblato Sands (44°49′88″N, 21°07′25″E) is a northeastern location, an arid area with scarce vegetation (*Orno-Quercetum cerris-virgiliane*), quite distinct from the other five localities. Derventa River Gorge (43°56′58″N, 19°21′27″E), Sicevo Gorge (43°19′55″N, 22°08′37″E), and Lazar

River Canyon (44°1′42″N, 21°57′28″E) are well-known glacial refugia of the central Balkans, have polydominant forests, endemic flora, and represent stable ecosystems where fluctuations in temperature are minimal.

Fifty to 100 female *Drosophila subobscura* were collected from each of the above localities. These females individually laid eggs [forming isofemale (IF) lines] under constant laboratory conditions (temperature 19°C, relative humidity ~60%, light 300 lux, and 12/12 h light/dark cycle) before being frozen (−20°C) and used for microsatellite fragment analysis. When F1 larvae appeared, the progeny of IF lines were used to determine maternal mitochondrial haplotypes, and males from the F1 were karyotyped for chromosomal inversion polymorphism.

Inversion polymorphism analysis

One F1 male from each isofemale line was individually crossed with 3–4 virgin females from the Küsnacht laboratory strain. This strain is homokaryotypic for all five acrocentric chromosomes of the set (A_{ST}, J_{ST}, U_{ST}, E_{ST}, and O_{ST}). Salivary glands from third-instar larvae were squashed and chromosomes stained with aceto-orcein solution. For the cytological analysis of gene arrangements, we used the chromosome map of Kunze-Mühl and Müller (1958). We stress here that both sets of chromosomes were analysed. To minimize any error in determining the karyotype of the crossed male, the chromosomes of eight third-instar larvae were analysed from the progeny of each cross. This reduced the probability of incorrect determination to $(1/2)^8$ for each chromosome. A *G*-test was used to identify any discrepancy in homogeneity of distributions of chromosomal arrangements between populations (Sokal and Rohlf, 1995), both for individual chromosomes and overall. The sequential Bonferroni test (Rice, 1989) was used to identify false-positive significant values due to multiple simultaneous testing.

Mitochondrial DNA extraction and digestion

We used a method described by Martinez *et al.* (1992) to obtain an enriched fraction of mtDNA. This fraction was digested with five restriction enzymes (EcoRI, EcoRV, HindIII, HaeIII, and HpaII), selected for their ability to detect mtDNA polymorphisms (Afonso *et al.*, 1990; Castro *et al.*, 1999). When rare restriction patterns were observed, double digestions were used to determine the exact position of the restriction sites. The different restriction patterns obtained (Fig. 1), using a given enzyme and the haplotypes, were named according to the notation of Latorre *et al.* (1992) and Castro *et al.* (1999). Digested fragments of mtDNA were separated on horizontal 0.8–1.2% gels. Before casting, ethidium bromide was added to gels in a final concentration of 0.1 $\mu g \cdot mL^{-1}$. λ DNA digested with HindIII and λ DNA double digested with HindIII-EcoRI were used as size standards. After electrophoresis, gels were photographed using a Bio-Rad Gel Doc 1000 (Bio-Rad Laboratories, Hercules, CA, USA).

To characterize the source of mtDNA variation within and between populations, we performed analysis of molecular variance (AMOVA). For population pairwise comparisons of haplotype diversity, we used the F_{ST} parameter, but modified for the haploid data set as heterozygosity data are not available. Arlequin (v.3.5.1.2) software was used (Excoffier and Lischer, 2010). Tajima's *D*-test (Tajima, 1989) was used to test for any departure from neutrality of the mtDNA haplotype distribution in populations. Population pairwise F_{ST} values are estimated based on mtDNA haplotypes.

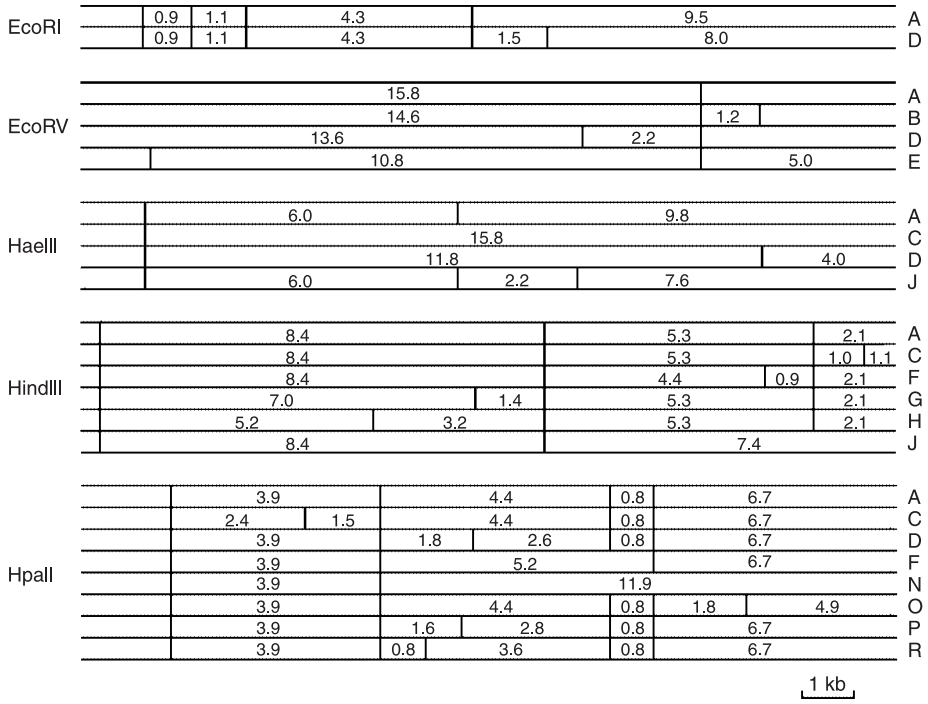

Fig. 1. Restriction patterns obtained with the five restriction enzymes used in the analysis of mtDNA in six *D. subobscura* populations. Fragment sizes are indicated in kilobase pairs.

DNA extraction and microsatellite analysis

We conducted a fragment analysis for 11 microsatellite loci (dsub05, dsub04, dsub18, dsub27, dsub20, dsub01, dsub03, dsub13, dsub19, dsub02, dsub15) following Pascual *et al.* (2000). Each chromosome was represented by two microsatellite loci (A: dsub05, dsub19; U: dsub03, dsub15; E: dsub13, dsub20; J: dsub18, dsub27), and by three microsatellite loci for the longest O chromosome (dsub01, dsub02, dsub04) (Santos *et al.*, 2010). Genomic DNA was extracted using a slightly altered version of the protocol of Martinez *et al.* (1992) – the alkaline treatment was excluded. Before the PCR reaction, the purity and concentration of DNA isolates were determined using an Eppendorf bio-photometer. The PCR was conducted in four multiplex reactions (dsub27, dsub20, and dsub04; dsub01, dsub18, and dsub05; dsub02, dsub13, and dsub19; dsub03 and dsub15). Primer pairs used to amplify microsatellites were as reported by Pascual *et al.* (2000). Four different fluorescent dyes (FAM, NED, PET, and VIC) were used to end-label one primer of each primer pair. The PCR conditions were similar to those of Pascual *et al.* (2001). A single soak at 95°C for 5 min was followed by 30 cycles of 1 min at 95°C, 30 s at 57°C, and 30 s at 72°C. The exception was a final elongation of 30 min at 60°C, with 2 μL (50 $\mu g \cdot \mu L^{-1}$) of DNA added to the total volume (20 μL) of the PCR mix. The GeneScan500-LIZ size standard was used as an internal size marker (Applied Biosystems). Fragment analysis was conducted on an ABI Prism 3130 automated sequencer. Only distinct and reproducible peaks were included in the genetic analysis. Data were analysed with the GeneMapper software (Applied Biosystems).

To assess the level of genetic diversity, we determined the mean number of alleles per locus for all loci (allelic richness – the number of alleles divided by the allelic range), allelic size range, and expected heterozygosity per locus versus all loci as standard diversity indices (Nei, 1987). The Garza-Williamson (GW) index (number of alleles divided by the allelic range) was computed for all populations (Garza and Williamson, 2001). This index is expected to be low in bottlenecked populations (GW index ranges between 0 and 1). All analyses were performed using Arlequin v.3.5 software (Excoffier and Lischer, 2010), and values obtained were assessed for significance using the non-parametric Mann-Whitney *U*-test with Past software (Hammer *et al.*, 2001).

To characterize the sources of genetic variation within and between populations, AMOVA was performed, using Wright's *F* statistics (Weir and Cockerham, 1984; Weir, 1996). The significance of the departure of the F_{ST} index from zero was done with 1023 permutations. Arlequin (v.3.5.1.2) software was used (Excoffier and Lischer, 2010).

In addition, we tested 11 microsatellite markers across six populations to determine whether selection was acting on loci. We used the F_{ST} method described in Cavalli-Sforza (1966) and Beaumont (2005) to evaluate the relationship between F_{ST} and expected heterozygosity (He). LOSITAN software (Beaumont and Nichols, 1996; Antao *et al.*, 2008) was used to implement the F_{ST} method and to test for outlier loci. The overall sample was analysed in two runs (for 15,000 simulations) under a stepwise mutation model. The first run optimized the baseline F_{ST} while the second run evaluated the outliers. We used that distribution of values to identify outlier loci that potentially had an excessively high or low F_{ST} value compared with the estimated baseline F_{ST}. LOSITAN can be downloaded for free from http://popgen.eu/soft/lositan/.

RESULTS

Inversion polymorphism variability

Table 1 shows the percentage of chromosomal arrangements for six *D. subobscura* populations. Several arrangements are found only in the two gorges and canyon, such as J_{3+4} and U_1 in the Sicevo Gorge, U_{1+2+8} in the Derventa River Gorge, U_{1+2+7} in the Lazar River Canyon, O_{3+4+7} in both the Sicevo Gorge and Lazar River Canyon, and E_{1+2} and O_{3+4+22} in all three populations. Heterozygosity is highest in the Botanical Gardens, intermediate and very similar among the two gorges and canyon, and the lowest in the Deliblato Sands population.

Table 2 gives the results of the non-parametric *G*-test, which compares variability in chromosomal arrangement between populations, for each of the five chromosomes and overall. Most of the differences are significant, with the exception of the comparisons between the Goc Mountain and Derventa River Gorge, Goc Mountain and Lazar River Canyon, Botanical Gardens and Deliblato Sands, Derventa River Gorge and Lazar River Canyon, and Sicevo Gorge and Lazar River Canyon populations. Analysing the six populations together, differences are significant for chromosomes U ($G = 32.270$, $P < 0.05$), E ($G = 67.702$, $P < 0.001$), O ($G = 71.366$, $P < 0.001$), and overall ($G = 199.322$, $P < 0.001$). For the distribution of arrangements on chromosome A (sex chromosome in *D. subobscura*) and chromosome J, none of the differences between populations are significant.

Table 1. Percentage of chromosomal arrangements in six *D. subobscura* populations

	Goc Mountain ($n = 60$)	Botanical Gardens ($n = 31$)	Deliblato Sands ($n = 33$)	Derventa River Gorge ($n = 88$)	Sicevo Gorge ($n = 85$)	Lazar River Canyon ($n = 47$)
A_{ST}	56.67	51.61	54.55	59.09	57.65	48.94
A_1	36.67	41.94	45.45	30.68	32.94	40.43
A_2	6.67	6.45	0.00	10.23	9.41	10.64
J_{ST}	19.17	41.94	24.24	32.39	23.81	18.09
J_1	80.83	58.06	75.76	67.61	75.00	81.91
J_{3+4}	0.00	0.00	0.00	0.00	1.19	0.00
U_{ST}	7.50	14.52	12.12	10.80	1.19	4.26
U_1	0.00	0.00	0.00	0.00	0.60	0.00
U_{1+2}	55.00	67.74	71.21	56.25	63.10	56.38
U_{1+2+6}	37.50	17.74	16.67	32.39	35.12	36.17
U_{1+2+8}	0.00	0.00	0.00	0.57	0.00	0.00
U_{1+2+7}	0.00	0.00	0.00	0.00	0.00	3.19
E_{ST}	25.83	35.48	28.79	39.77	26.79	15.96
E_{1+2}	0.00	0.00	0.00	3.41	4.76	7.45
E_{1+2+9}	50.00	25.81	18.18	41.48	30.36	51.06
$E_{1+2+9+12}$	00.00	1.61	0.00	0.00	0.00	0.00
E_8	24.17	37.10	53.03	15.34	38.10	25.53
O_{ST}	12.50	46.77	36.36	18.75	11.90	17.02
O_{3+4}	46.67	40.32	37.88	53.98	61.90	55.32
O_{3+4+1}	33.33	11.29	19.70	14.77	11.31	12.77
O_{3+4+2}	6.67	1.61	6.06	5.68	1.19	3.19
O_{3+4+22}	0.00	0.00	0.00	6.25	13.10	7.45
O_{3+4+7}	0.00	0.00	0.00	0.00	0.60	3.19
O_6	0.83	0.00	1.67	0.57	0.00	1.06
H	49.17	58.87	48.40	53.19	53.27	53.19
IFR	84.13	81.58	85.11	83.25	83.65	82.65

mtDNA haplotype variability

Two haplotypes (I and II) are high in frequency in all populations and rare haplotypes are present across all populations in low frequencies (Table 3). The frequency of haplotype I is the lowest (25.81%) in the Derventa River Gorge population, intermediate and similar in the Botanical Gardens, Goc Mountain, and Sicevo Gorge populations, and highest in the Lazar River Canyon (52.63%) and Deliblato Sands (50.72%) populations. The frequency pattern of haplotype II is the opposite across populations. Rare haplotypes are the most frequent in the Botanical Gardens (10.91%) and Sicevo Gorge populations (9.45%). Haplotype diversity is highest in the Deliblato Sands population (0.585 ± 0.034) and lowest in the Derventa River Gorge population (0.436 ± 0.057).

Table 2. Variability of chromosomal arrangement frequencies among six *D. subobscura* populations

Pop.	*G*			BG			DS			DRG			SG		
	Chromosome	*G*-stat	*P*												
BG	A	0.243													
	J	10.426													
	U	8.715													
	E	9.773													
	O	30.239	***												
	all	59.407	***												
DS	A	0.324		A	0.002										
	J	0.652		J	4.568										
	U	9.510		U	0.215										
	E	22.499	**	E	3.097										
	O	14.650		O	4.103										
	all	47.720	***	all	12.046										
DRG	A	0.951		A	1.434		A	1.247							
	J	6.506		J	1.810		J	1.553							
	U	1.397		U	5.312		U	6.490							
	E	8.138		E	12.643		E	33.302	**						
	O	13.186		O	16.244		O	8.917							
	all	30.384		all	37.595	*	all	51.791	**						
SG	A	0.475		A	0.897		A	0.795		A	0.114				
	J	0.996		J	6.709		J	0.001		J	2.902				
	U	8.365		U	19.591	**	U	18.094	**	U	15.990	*			
	E	11.092		E	1.424		E	5.170		E	24.992	**			
	O	24.272	**	O	25.499	**	O	23.842	**	O	14.095				
	all	46.070	**	all	54.765	***	all	48.510	***	all	58.110	***			
LRC	A	0.884		A	0.418		A	0.000		A	1.418		A	0.941	
	J	0.041		J	10.508		J	0.889		J	6.590		J	1.294	
	U	0.935		U	10.050		U	10.051		U	3.664		U	2.770	
	E	2.208		E	13.506		E	22.772	***	E	18.770	*	E	13.533	
	O	12.058		O	12.807		O	9.522		O	1.404		O	7.246	
	all	16.570		all	47.640	**	all	43.617	**	all	31.905		All	25.833	

Note: G = Goc Mountain, BG = Botanical Gardens, DS = Deliblato Sands, DRG = Derventa River Gorge, SG = Sicevo Gorge, LRC = Lazar River Canyon. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$ (after Bonferroni correction).

Table 3. Percentage of isofemale lines (IF) with different haplotypes and their restriction patterns in six *D. subobscura* populations

Haplotypes	G	BG	DS	DRG	SG	LRC	Restriction patterns				
							EcoRI	EcoRV	HaeIII	HindIII	HpaII
I	29.66	27.27	50.72	25.81	32.43	52.63	A	A	A	A	A
II	65.25	61.81	40.58	70.97	58.11	42.11	A	A	C	A	A
III	0.85	/	1.45	/	/	2.63	A	A	A	A	F
IV	/	3.64	/	1.61	/	/	A	A	C	A	D
V	1.69	1.82	/	/	/	/	A	A	C	A	P
VI	/	/	/	/	2.70	/	A	B	C	A	A
VII	/	/	/	/	2.70	/	A	A	C	A	R
VIII	0.85	/	/	/	/	/	D	A	C	A	A
IX	/	/	1.45	/	/	/	A	D	C	A	A
X	/	/	1.45	/	/	/	A	E	A	A	A
XI	/	/	1.45	/	/	/	A	A	A	F	A
XII	0.85	/	/	/	/	/	A	A	A	H	A
XIII	/	/	/	/	1.35	/	A	A	C	C	A
XIV	/	/	1.45	/	/	/	A	A	C	G	A
XV	0.85	/	/	/	/	/	A	A	C	J	A
XVI	/	/	1.45	/	/	/	A	A	D	A	A
XVII	/	/	/	1.61	/	/	A	A	J	A	A
XVIII	/	/	/	/	/	2.63	A	A	C	A	C
XIX	/	1.82	/	/	/	/	A	A	C	A	N
XX	/	1.82	/	/	/	/	A	A	C	A	O
XXI	/	1.82	/	/	/	/	A	A	A	A	O
XXII	/	/	/	/	1.35	/	A	A	A	A	P
D*	/	/	/	/	1.35	/	A	A	A	A	A
Total IF	118	55	69	62	74	38					
Haplotype diversity	0.49 ± 0.037	0.551 ± 0.059	0.585 ± 0.034	0.436 ± 0.057	0.554 ± 0.040	0.559 ± 0.041					

Note: G = Goc Mountain, BG = Botanical Gardens, DS = Deliblato Sands, DRG = Derventa River Gorge, SG = Sicevo Gorge, LRC = Lazar River Canyon. Numbers were designated to the rare haplotypes starting from the most frequent one. *Haplotype D has the same restriction pattern as haplotype I, but harbours a large insertion.

The results of *F*-statistics are given in Table 4. The Deliblato Sands and Lazar River Canyon populations differ significantly from all other populations, while the F_{ST} value between them is not significantly different from zero ($F_{ST} = -0.0186$; $P = 0.999$). Analysis of molecular variance found that 96.78% of total mtDNA variability is explained by within-population variation, while 3.22% is due to between-population variation. Although small, the between-population variation is the cause of significant differentiation between populations ($F_{ST} = 0.0322$, $P = 0.00098$).

In Tajima *D*-tests, negative values were obtained for all the populations analysed (Table 5). The *D*-values were not significantly different from zero for each population ($P > 0.05$), or when the two gorges and the canyon were grouped together ($D = -1.4549$; $P > 0.05$). A significant difference was obtained when haplotypes from all populations were combined ($D = -1.952$; $P < 0.01$), and when the Botanical Gardens, Goc Mountain, and Deliblato Sands populations were grouped together ($D = -1.79140$, $P < 0.01$).

Microsatellite variability

The mean values of allelic richness per locus ranged between 12.182 and 16.364 (Table 6). The highest value was recorded in the Deliblato Sands population and the lowest in the Derventa River Gorge population. Significant differences in allelic richness were found between the Goc Mountain and Derventa River Gorge populations (Mann-Whitney *U*: 14.454 vs. 12.182, $Z = -2.024$, $P = 0.04$), between the Botanical Gardens and Derventa River Gorge populations (Mann-Whitney *U*: 16.182 vs. 12.182, $Z = -2.352$, $P = 0.015$), between the Deliblato Sands and Derventa River Gorge populations (Mann-Whitney *U*: 16.364 vs. 12.182, $Z = -2.68$, $P = 0.005$), and between the Derventa River Gorge and Lazar River Canyon populations (Mann-Whitney *U*: 12.182 vs. 15.818, $Z = -2.707$, $P = 0.005$).

The allelic size range for the six populations is given in Table 7. Mean allelic size ranged between 17.81 and 25.54. A significant difference was obtained only between the Deliblato Sands and Sicevo Gorge populations (Mann-Whitney *U*: 23.0 vs. 18.0, $Z = -2.076$, $P = 0.034$). In addition, the Garza-Williamson (GW) index (number of alleles divided by the allelic range) was computed for all populations (results not shown). This index is expected to be low in bottlenecked populations (GW index ranges between 0 and 1). The GW index is sensitive to population bottlenecks, and should be near 0 for populations that have passed through a bottleneck, but close to 1 in stationary populations (Garza and Williamson, 2001; Williamson-Natesan, 2005). All populations had a high index (0.56–0.92), suggesting that none has experienced a recent bottleneck.

The mean expected heterozygosity (Table 8) is quite high in all six populations (range 0.84045–0.87344). Expected heterozygosity was highest in the Botanical Gardens population and lowest in the Lazar River Canyon population. However, no significant differences in expected heterozygosity were detected among the six populations. Analysis of molecular variance showed that 2.81% of total microsatellite variability is explained by within-population variation, while 0.65% is due to within-population variation among individuals. No significant variation between populations was obtained ($F_{ST} = 0.0065$; $P = 0.2004$). Population pairwise F_{ST} values based on the number of different alleles (Table 9) generally showed a significant difference between the Botanical Gardens population and the other five, while populations from the canyon and two gorges were different from the other three, with no significant differences among them.

Table 4. Population pairwise F_{ST}-values based on mtDNA haplotypes

	G		BG		DS		DRG		SG	
Populations	F_{ST}	P	F_{ST}	P	F_{ST}	P	F_{ST}	P	F_{ST}	P
BG	−0.0091	0.7022								
DS	0.0828	0.0000***	0.0697	0.0059**						
DRG	−0.0061	0.5391	−0.0064	0.4932	0.1200	0.0010**				
SG	−0.0021	0.3779	−0.0068	0.5020	0.0393	0.0391*	0.1690	0.1563		
LRC	0.0818	0.0156*	0.0693	0.0332*	−0.0186	0.9990	0.0098	0.0049**	0.0366	0.0654

Note: Conventional *F*-statistics are used. G = Goc Mountain, BG = Botanical Gardens, DS = Deliblato Sands, DRG = Derventa River Gorge, SG = Sicevo Gorge, LRC = Lazar River Canyon. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$.

Table 5. mtDNA haplotype frequencies, properties of variability, and results of Tajima's *D*-tests in six *D. subobscura* populations

Populations	I	II	Rare	Haplotype diversity	Tajima *D*	Tajima *P*
G	29.66	65.25	5.08	0.490 ± 0.037	−1.15	0.126
BG	27.27	61.81	10.91	0.551 ± 0.059	−1.22	0.106
DS	50.72	40.58	8.70	0.585 ± 0.034	−1.34	0.078
DRG	25.81	70.97	3.23	0.436 ± 0.057	−0.52	0.348
SG	32.43	58.11	9.45	0.554 ± 0.040	−0.88	0.229
LRC	52.63	42.11	5.26	0.559 ± 0.041	−0.31	0.414
DRG + SG + LRC	34.48	59.20	6.32	0.529 ± 0.025	−1.46	0.059
G + BG + DS	35.12	57.44	7.44	0.549 ± 0.021	–1.79	0.008**
Overall	34.85	58.17	6.97	0.539 ± 0.016	−1.95	0.001**

Note: G = Goc Mountain, BG = Botanical Gardens, DS = Deliblato Sands, DRG = Derventa River Gorge, SG = Sicevo Gorge, LRC = Lazar River Canyon. $^{**}P < 0.001$.

Table 6. Mean number of alleles per locus/all loci (allelic richness)

	Populations							
Loci	G	BG	DS	DRG	SG	LRC	mean	s.d.
dsub05	14	16	20	14	16	17	15.857	2.268
dsub04	17	20	19	17	20	21	18.571	1.902
dsub18	15	16	16	13	16	16	15.286	1.604
dsub27	18	19	19	12	16	16	17.000	2.708
dsub20	16	21	23	16	14	18	17.714	3.251
dsub01	14	13	12	11	14	14	13.143	1.345
dsub03	12	10	14	9	10	13	11.429	1.902
dsub13	14	10	14	10	12	15	12.714	2.138
dsub19	10	17	17	10	12	14	12.857	3.237
dsub02	15	19	12	10	11	12	13.429	3.552
dsub15	14	17	14	12	11	18	14.286	2.752
mean	14.454	16.182	16.364	12.182	13.818	15.818	14.753	1.691
s.d.	2.442	3.763	3.557	2.601	2.994	2.601	2.888	0.576

Note: G = Goc Mountain, BG = Botanical Gardens, DS = Deliblato Sands, DRG = Derventa River Gorge, SG = Sicevo Gorge, LRC = Lazar River Canyon.

The software LOSITAN identified no outlier loci in the overall sample, suggesting neutrality of the microsatellites analysed. No differences were observed between markers located below the neutral area (candidates for being under balancing selection) and markers located above the neutral area (candidates for being under directional selection).

DISCUSSION

During glacial–interglacial periods, it is thought that species retreated into refugia, which provided stable conditions for long-term persistence in biodiversity, and the subsequent

Table 7. Allelic size range

	Populations							
Loci	G	BG	DS	DRG	SG	LRC	mean	s.d.
dsub05	18	17	25	23	18	31	22.286	6.601
dsub04	27	35	28	30	23	25	27.714	4.680
dsub18	22	58	25	15	15	17	23.429	15.884
dsub27	25	25	24	22	21	26	24.000	2.160
dsub20	23	24	27	29	18	21	23.571	3.645
dsub01	14	21	12	10	15	23	15.714	4.751
dsub03	18	10	16	9	16	16	14.571	4.756
dsub13	21	14	32	21	27	32	24.000	6.658
dsub19	13	25	26	11	16	16	17.143	6.644
dsub02	21	24	21	12	12	12	17.429	6.425
dsub15	20	28	17	16	15	24	19.857	4.741
mean	20.181	25.545	23.000	18.000	17.818	22.091	20.883	3.262
s.d.	3.849	12.739	5.916	7.470	4.309	6.395	6.769	2.932

Note: G = Goc Mountain, BG = Botanical Gardens, DS = Deliblato Sands, DRG = Derventa River Gorge, SG = Sicevo Gorge, LRC = Lazar River Canyon.

Table 8. Mean expected heterozygosity per locus and population

	Populations							
Loci	G	BG	DS	DRG	SG	LRC	mean	s.d.
dsub05	0.91322	0.91045	0.91731	0.91478	0.92081	0.89367	0.91193	0.01087
dsub04	0.86480	0.88538	0.88557	0.86048	0.93355	0.90710	0.88597	0.03397
dsub18	0.85698	0.88813	0.85891	0.90045	0.90803	0.89291	0.88034	0.02512
dsub27	0.88971	0.85884	0.89308	0.86878	0.89430	0.88474	0.88274	0.01896
dsub20	0.81241	0.91100	0.89614	0.91176	0.80096	0.80749	0.85031	0.05523
dsub01	0.88343	0.86548	0.87606	0.88311	0.88581	0.89008	0.88106	0.00815
dsub03	0.82351	0.85358	0.81789	0.70857	0.78289	0.74957	0.79460	0.05074
dsub13	0.73490	0.73004	0.63171	0.71011	0.62785	0.59250	0.68029	0.08389
dsub19	0.88036	0.89684	0.88993	0.88475	0.90130	0.90854	0.89173	0.01161
dsub02	0.88950	0.91672	0.82918	0.87020	0.83688	0.83745	0.86691	0.03773
dsub15	0.88912	0.89133	0.87918	0.81294	0.85411	0.88092	0.86370	0.02961
mean	0.85799	0.87344	0.85227	0.84781	0.84968	0.84045	0.85360	0.03326
s.d.	0.05062	0.05207	0.07877	0.07391	0.08814	0.09569	0.06483	0.02293

Note: G = Goc Mountain, BG = Botanical Gardens, DS = Deliblato Sands, DRG = Derventa River Gorge, SG = Sicevo Gorge, LRC = Lazar River Canyon.

expansions would have resulted in a reduced genetic variability in other populations in the range (Hewitt, 2004). The three main southern refugia during the Pleistocene glaciations in Europe are the Iberian, Italian, and Balkan peninsulas (Bennett *et al.*, 1991; Hewitt, 1996). Canyons and gorges in the southern and central part of the Balkan Peninsula show marked floristic diversity and a polydominant type of forest with endemic plant species (Misic, 1981). In

Table 9. Population pairwise F_{ST}-values (number of different alleles)

	G		BG		DS		DRG		SG	
Populations	F_{ST}	P	F_{ST}	P	F_{ST}	P	F_{ST}	P	F_{ST}	P
BG	0.0059	0.002**								
DS	0.0008	0.289	0.0089	0.000***						
DRG	0.0068	0.023*	0.0134	0.000***	0.0064	0.072				
SG	0.0061	0.002**	0.0197	0.000***	0.0056	0.009**	0.0056	0.094		
LRC	0.0060	0.001***	0.0170	0.000***	−0.0008	0.649	0.0033	0.198	0.0020	0.207

Note: G = Goc Mountain, BG = Botanical Gardens, DS = Deliblato Sands, DRG = Derventa River Gorge, SG = Sicevo Gorge, LRC = Lazar River Canyon. Significance based on 1023 permutations: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

addition, the continuous presence of water, as a regulator and accumulator of heat, buffers changes in temperature. Nevertheless, the locations sampled in this study differed in terms of microclimate and vegetation characteristics.

In the present study, we aimed to identify glacial refugia within the central Balkans, by identifying specific patterns of variability using an integrative approach that simultaneously analyses three kinds of genetic markers (chromosomal and molecular markers such as mitochondrial and microsatellite DNA) on six *D. subobscura* populations.

In general, the results for inversion polymorphism parameters (heterozygosity, index of free recombination) in the six populations analysed are similar to those of other *D. subobscura* populations in the Central Balkans (Krimbas, 1993; Zivanovic *et al.*, 2002; Andjekovic *et al.*, 2007; Stamenkovic-Radak *et al.*, 2008).

The inversion polymorphism results indicate highly structured *D. subobscura* populations. In the populations from the canyon and two gorges, several chromosomal arrangements are present in low frequencies, which are absent in the other three populations. Among these, the arrangements J_{3+4} in Sicevo Gorge and U_{1+2+7} in Lazar River Canyon are characteristic of the southern parts of the *D. subobscura* range, such as Greece and Asia Minor. Some arrangements (E_{1+2} and O_{3+4+22}) persist in gorges and canyons compared with other populations where they are found only occasionally (Zivanovic *et al.*, 2002; Kalajdzic *et al.*, 2006; Zivanovic and Mestres, 2011). Similar to these, the arrangement O_{3+4+7} was found in another gorge, Djerdap (Zivanovic, 2007). Their frequency is slightly higher and corresponds to the southern parts of the species range. In addition to historical processes, favourable and mild climatic conditions, together with possible minor fluctuations in population size may be responsible for the preservation of rare chromosomal arrangements. Since inversion polymorphism of *D. subobscura* is an adaptive genetic marker (Prevosti *et al.*, 1988; Orengo and Prevosti, 1996; Pegueroles *et al.*, 2010), greater habitat differentiation in these locations provides the opportunity for conservation of chromosomal arrangements that are absent in other areas. The marked similarities between the Sicevo Gorge and Lazar River Canyon populations, but not the Derventa River Gorge population, are probably due to the similar microecological characteristics of these localities, topographic position of sampling sites, and the sampling season (June vs. September).

The frequency pattern of particular chromosomal variants in *D. subobscura* populations from gorges and canyons suggests these habitats in the Balkan Peninsula may have been

significant refugia or stepping-stones in the migration route in the recolonization of Europe. On the other hand, gorges and canyons, although representing areas with conserved topography are not isolated from surrounding populations. The dispersion capacity of *D. subobscura* is high (Ayala *et al.*, 1989), and gene flow could to a great extent mask the effects of historical adaptive processes and lead to similarity among populations in different habitats. Furthermore, *D. subobscura* recently colonized South and North America, where it has adapted very rapidly and, as a consequence, has developed clines for some markers of genetic variability, such as chromosomal arrangements (Prevosti *et al.*, 1988, 1989; Pascual *et al.*, 1993), similar to those found in Old World populations.

Variability of mtDNA haplotypes in the six populations examined corresponds to that observed in other parts of *D. subobscura*'s range across Europe and the New World, with two dominant haplotypes whose presence seems to be stable and probably older than the last glacial period (Latorre *et al.*, 1986, 1992; Afonso *et al.*, 1990; Rozas *et al.*, 1990; Moya *et al.*, 1993; Pinto *et al.*, 1997; Castro *et al.*, 1999). In this respect, *D. subobscura* populations of the Balkan Peninsula contributed to the recolonization of Europe, but based on mtDNA variability we cannot confirm it as a refugium in its own right, as shown previously by Krimbas (1993) based on inversion polymorphism and confirmed in the present study.

Analysis of mtDNA variability did not reveal a higher degree of genetic diversity in the canyon and two gorges compared with the other three locations. Haplotype diversity was greatest in the Sicevo Gorge population, and lowest in the Derventa River Gorge population. It is possible that historical and adaptive processes act to increase diversity in refugium-like habitats, but high gene flow, characteristic of *D. subobscura*, masks the resulting effect (Ayala *et al.*, 1989). Of the other three populations, that of the Deliblato Sands, which did not show the characteristics of a refugium, had the highest haplotype diversity, while the Botanical Gardens population had the highest frequency of rare haplotypes. The latter can be explained by the strong anthropogenic impact on this population and number of ecological niches (Valiati and Valente, 1996).

The F_{ST} analysis showed that the Deliblato Sands and Lazar River Canyon populations are similar but significantly differentiated from the other four. The ratio of the frequencies of the two dominant haplotypes influences this structure, as only in these two populations does haplotype I occur at a higher frequency than haplotype II. This was probably a result of spatial and temporal differences in sampling. The significance of environmental changes in shaping mtDNA variability in *D. subobscura* has been shown previously (Christie *et al.*, 2010), in that haplotype I was most frequent in optimal conditions for this species, in June, and haplotype II was most frequent in unfavourable conditions of high larval density.

The appearance of a haplotype in a particular area is not proof that it originated there, but it could also be in non-random association with some of the chromosomal gene arrangements. Disequilibria between chromosomal arrangements and the two most frequent mtDNA haplotypes were reported by Oliver *et al.* (2002) in a natural population from the Island of Majorca (Spain), while our recent study on populations from the Derventa and Sicevo gorges (Jelic *et al.*, 2012) reveals the absence of linkage disequilibrium between mitochondrial haplotypes and chromosomal inversion arrangements in these populations. Its presence may depend on evolutionary mechanisms whose actions are temporally or spatially different depending on ecological, geographical, and historical processes.

The negative values recorded for the Tajima *D*-test for each of the six populations in the present study are in line with most mtDNA population studies for this species. Negative

values are likely influenced by an excess of haplotypes with low frequencies, which could be explained by these populations experiencing bottlenecks and rapid expansions afterwards. The Tajima *D*-values were non-significant except when haplotypes were based on all six populations together, or the three populations other than the two gorges and the one canyon. It is possible that in the gorges and canyon the bottlenecks were less severe, and there were no marked expansions afterwards as in the other three populations.

According to our results, the pattern of microsatellite variability of these six populations is in line with that of European populations (Pascual *et al.*, 2001) if we take into account the expected heterozygosity, but standard diversity indices (allelic richness per locus, allelic size range) for the Balkan populations are slightly lower than in other parts of Europe. The Botanical Gardens and Deliblato Sands populations fit the European pattern of microsatellite variability, followed by Goc Mountain and Lazar River Canyon; lower expected heterozygosity, allelic richness per locus, and allelic size range were observed for the two populations collected from very specific habitats (Derventa River Gorge and Sicevo Gorge). The steep cliffs of these gorges climatically isolate the gorge floors as relatively moist and warm habitats. In addition, the continuous presence of water, as a regulator and accumulator of heat, buffers changes in temperature. This is the case in both the Derventa River Gorge and Sicevo Gorge populations, where more stable weather conditions might not favour the heterozygotes. On the other hand, specific microhabitat in the Botanical Gardens population consists of a range of different ecological niches, and different genotypes may have adapted to each of the different niches. Furthermore, that might also be the cause of the high heterozygosity detected in this population. Habitats generally presumed to be refugia, such as the two gorges and canyon, do not group according to their microsatellite variability; on the contrary, two of them have lower expected heterozygosity and other diversity parameters. Microsatellite differentiation among the six populations in the present study was not significant. This is consistent with reports for other European populations (Pascual *et al.*, 2001) and is expected, given the known high migration rate of *D. subobscura* (Taylor *et al.*, 1984). Taking these data into account, the Balkan Peninsula was unlikely to have been a glacial refugium for *D. subobscura*.

Microsatellite loci are highly polymorphic markers but selectively neutral, distributed throughout the nuclear genome, and the characterization of particular markers in *D. subobscura* confirms their high variability (Pascual *et al.*, 2000, 2001). No genetic differentiation among European populations was detected, indicating that gene flow is high and the microsatellites used in those studies represent neutral markers, not subject to differentiation due to selection. The results of microsatellite variability in the present study show no significant departures from neutrality for any of the loci tested in the populations. In analysing a different set of populations (two from Goc Mountain, Botanical Gardens, Derventa Gorge, and Sicevo Gorge), Kurbalija Novicic *et al.* (2011) showed weak departures of neutrality in the gorge populations for the same loci. The discrepancy between these results could be due to different sample sizes and the different populations analysed. This can be overcome by different ecological sorting on the landscape and individual analyses combining various genetic markers from the same individual.

The results obtained with inversion polymorphism as a marker suggest that populations in different habitats should be subjected to habitat-specific selection regimes. Also, results obtained on chromosomal polymorphism of this species suggest that it may be suitable for monitoring climate change (Rodriguez-Trelles and Rodriguez, 1998). The co-adaptation hypothesis states that different alleles of genes will be present in different gene arrangements and

selection acts upon them (Dobzhansky, 1948; Aquadro *et al.*, 1994; Hoffmann *et al.*, 2004). Even though microsatellite loci are theoretically considered to be selectively neutral, selection may influence the variability of a microsatellite locus, either itself having an important function in the genome or, alternatively, the microsatellite may be influenced by selective processes that act on a linked nucleotide site. As both processes predict lower levels of neutral polymorphism in regions of low as opposed to high recombination, the observed microsatellite variability should reflect this pattern. In this sense, further molecular investigations of particular inversion gene arrangements in *D. subobscura* populations are merited, with microsatellite markers taken from the same individuals.

According to some authors, the genetic similarity of populations could be a result of isolation in their local habitats and small population size, instead of high gene flow (Greenbaum *et al.*, 1978). In contrast, our results for *D. subobscura* chromosome markers and mtDNA to some extent suggest adaptive population divergence and local adaptation to specific microhabitats, despite high gene flow. Microsatellite data suggest that the effective population size of all tested natural populations of *D. subobscura* is extremely large (Pascual *et al.*, 2001; Z. Kurbalija Novicic *et al.*, unpublished data). Under such conditions, selection is effective in shaping the evolution of a population. The adaptive population divergence seen in our populations of *D. subobscura* indicate that local adaptations at the molecular level can occur despite high gene flow and large effective sizes. Although microsatellite variability analysis of the populations in this study did not confirm any recent size reduction and mtDNA haplotype variability indicates such events, it should be noted that mtDNA markers are four times more sensitive to reduction in population size. Population size fluctuates, as do factors such as gene flow, so it is difficult to determine with confidence how the pattern at any one time arose and how it is representative.

There is no reason to doubt that the gorges and canyons of the Balkan Peninsula, considered refugia based on paleobotanical evidence, were favourable habitat for insects such as *D. subobscura*, with its strong potential for colonizing and adapting to different conditions. The genomic signatures analysed in this study mostly reveal high adaptive divergence and gene flow and the absence of recent bottlenecks. The pattern of colonization is either rapid from several southern refugia or slower, more steady expansion when refugial genomes spread more equally. All of these range changes will of course be affected by the local and regional topography and, in that sense, the populations from the Balkan mountains and islands (in Greece), together with gorges and canyons analysed in this paper should be included.

By integrating data from multiple neutral and adaptive genetic markers, it is possible to describe to some extent the history and demography of the population genetic diversity of *D. subobscura* and other species. Such studies are rare (Bos *et al.*, 2008) but they provide complex and valuable information on the evolutionary history of populations and help to predict their future fate.

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