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Microsatellite variability of *Drosophila subobscura* populations from the central Balkans

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ABSTRACT

Background: Some populations of *Drosophila subobscura* (Collin) inhabit geographically separate and ecologically distinct habitats of the central Balkans.

Hypothesis: Some of the separate populations were genetically isolated in glacial refugia and have subsequently diverged, leading to high genetic diversity.

Methods: We sampled five different *D. subobscura* populations. Using fragment analysis for 11 microsatellite loci, we used standard diversity parameters (expected heterozygosity, allelic richness, allele size range) to estimate population genetic structure and genetic diversity.

Results: We found significant differences in the number of alleles, range of allele sizes, and expected heterozygosity between populations from ecologically distinct microhabitats. However, their geographical distance from each other did not contribute to their genetic differences. Analysis of molecular variance showed slight inter-population differentiation ($F_{ST} = 0.00996$, $P = 0.0215$).

Conclusions: Microsatellite variability parameters generally match those of other European populations. *Drosophila subobscura* populations of the Balkan Peninsula likely did not remain isolated in glacial refugia. Instead, our results indicate high levels of gene flow and local divergence at the molecular level.

Keywords: adaptive evolution, chromosomal inversions, genetic diversity, microhabitats.

INTRODUCTION

Drosophila subobscura has a broad Palearctic distribution (Krimbas, 1993). It occurs throughout Europe and reaches its southern limit in Morocco. It also occurs on some Atlantic islands and recently colonized South and North America (Ayala *et al.*, 1989; Rozas *et al.*, 1990).

Drosophila subobscura has been investigated extensively in the past: numerous studies have focused on population genetic structure screening of different natural populations,

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based on mtDNA analysis (Afonso *et al.*, 1990; Latorre *et al.*, 1992; García-Martínez *et al.*, 1998; Castro *et al.*, 1999), allozymes (Marinkovic *et al.*, 1978; Pinto *et al.*, 1997; Castro *et al.*, 1999), and inversion polymorphism (Prevosti, 1974; Prevosti *et al.*, 1984; Brehm and Krimbas, 1987; Krimbas, 1993; Khadem *et al.*, 1998; Zivanovic *et al.*, 2002; Andjekovic *et al.*, 2007; Zivanovic, 2007; Kenig *et al.*, 2010). The results of these studies suggest that different markers show different amounts of geographic variation. Chromosomal arrangements are highly differentiated among European populations, but mtDNA and allozymes are not.

Pascual *et al.* (2000) were the first to analyse microsatellite variability in natural populations of *D. subobscura*. The mean length of the repeat unit of microsatellite clones was 15, higher than that observed in other *Drosophila* species. The level of microsatellite variation, measured as variance in repeat number and heterozygosity, was similar to that of *D. pseudoobscura*, and higher than that of *D. melanogaster* and *D. simulans*. Their data suggest that higher levels of microsatellite variation – and possibly density – in *D. subobscura* compared with *D. melanogaster*, are due both to higher average effective population size and higher intrinsic slippage rate in *D. subobscura*.

Pascual *et al.* (2001) also performed a comparative study (10 microsatellite loci) of five European and two North American populations. No genetic differentiation among European populations was detected, indicating that gene flow is high and that the microsatellites used in their work represent neutral markers, not subject to differentiation due to selection. The F_{ST} values did not show significant correlations between genetic differentiation and geographic distance among populations. Pascual *et al.* also found greater genetic diversity in the mean number of alleles per locus (between 18 and 36 alleles per locus) among European populations relative to American (between 5.3 and 5.5 alleles per locus) populations. They also reported a high expected heterozygosity for all loci (between 0.843 and 0.891) in all European populations. In all the European populations tested, no significant linkage disequilibrium between pairs of microsatellite loci was observed. The results were consistent with a two-phase microsatellite mutational model, indicating that most alleles are generated by gain or loss of a repeat unit, but that some alleles originate from more complex mutations.

Here, we focus on genetic diversity patterns of *D. subobscura* populations in the Balkan Peninsula (central Balkans), which was one of the main European refugia for many European species during the last 2 million years of glacial–interglacial climate oscillations (Hewitt, 2000). Thus the specific natural habitats now found in the region were probably shaped by glacial–interglacial climate oscillations. The inversion chromosomal polymorphism data of *D. subobscura* suggest that the Balkan Peninsula may have played an important role in repopulating Europe after the last glaciation (Menozzi and Krimbas, 1991; Krimbas, 1993; Zivanovic, 2007).

Microsatellite loci are highly polymorphic markers, distributed throughout the nuclear genome and generally neutral unless linked to loci under strong selection. Characterization of these markers in *D. subobscura* confirms their high variability (Pascual *et al.*, 2000; Santos *et al.*, 2010) and therefore makes them good candidates for a study of population genetic structure. Multi-locus approaches may be useful for detecting the regions that depart from the neutrality pattern and that are under certain selection pressure.

We focus on microsatellite analysis of five *D. subobscura* populations from the central Balkans. Sampled populations differ significantly in chromosomal arrangements (Kenig *et al.*, 2010) and occupy geographically and ecologically different habitats. Our aims were: (1) to assess the level of genetic diversity within and between populations, and the genetic

differentiation among populations from the central Balkans region; and (2) to analyse the present data in the context of other European populations.

MATERIALS AND METHODS

Population samples

Drosophila subobscura flies were sampled at five localities in Serbia: beechwood (B), oakwood (O), Botanical Gardens (BG), Derventa River Gorge (D), Sicevo Gorge (S). The beechwood (43°33′28.43″N, 20°45′10.96″E) and oakwood (43°32′57.38′N, 20°40′2.32″E) are both in Mountain Goc (Central Serbia). These habitats are close to each other (~6 km apart), but nonetheless have distinct microclimates. The beechwood is more humid and has dense vegetation coverage, whereas the oakwood has less dense vegetation and is slightly warmer. The third locality, the Botanical Gardens (44°49′N, 20°28′E) in the central, urban part of Belgrade, has a microclimate affected by high anthropogenic activity. Derventa River Gorge (43°56′58″N, 19°21′27″E) and Sicevo Gorge (43°19′55″N, 22°08′38″E) are central Balkans gorges that are well known glacial refugia, have endemic flora, and represent stable ecosystems where fluctuations in temperature are minimal.

We collected 51, 38, 47, 43, and 48 wild-caught inseminated females from the oakwood, beechwood, Botanical Gardens, Derventa Gorge, and Sicevo Gorge, respectively. These females laid eggs 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.

DNA extraction and microsatellite analysis

To analyse the genetic structure of different populations of *D. subobscura* from the Balkan Peninsula, we conducted a fragment analysis for 11 microsatellite loci (dsub05, dsub04, dsub18, dsub27, dsub20, dsub01, dsub03, dsub13, dsub19, dsub2, 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, dsub2, 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 biophotometer. 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 in Pascual *et al.* (2000). Four different fluorescent dyes (FAM, NED, PET, and VIC) were used to end-lable 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 that a final elongation of 30 min at 60°C was included, and 2 μL (50 $\mu g \cdot \mu L^{-1}$) of DNA were 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, well-marked amplified peaks were included in the genetic analysis. Data were analysed with the GeneMapper software (Applied Biosystems).

Statistical analysis

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). We also computed the molecular diversity index, which assumes average gene diversity over loci, performed for each population (Tajima, 1983, 1993; Nei, 1987; Ewens, 1972; Zouros, 1979). In addition, 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 *et al.*, 2005), and values obtained were assessed for significance using the non-parametric Mann-Whitney *U*-test with Past software (Hammer *et al.*, 2001). For each data set generated, using the Bottleneck (v.1.2) program, we performed a Wilcoxon sign-rank test to determine whether a significant number of loci featured excess heterozygosity: this would indicate a recent bottleneck event, assuming a two-phase mutation model [TPM (Cornuet and Luikart, 1996)]. Pascual *et al.* (2001) showed that this set of microsatellite loci of *D. subobscura* provides the best power to detect a recent bottleneck, if the loci evolved via a TPM.

Linkage disequilibrium among microsatellite loci was assessed using Arlequin v.3.5 (Excoffier *et al.*, 2005). We included a permutation test using the EM algorithm with 10,000 permutations. In addition, because of the large number of tests, we applied the sequential Bonferroni test (Rice, 1989). We also used a Bayesian approach (BayeScan 2.0) to detect loci under selection. F_{ST} values reflect contributions from locus-specific effects (beta) such as selection, and population-specific effects (alpha) such as genetic drift and immigration rates. BayeScan estimates the locus and population effects on these F_{ST} values using a hierarchical approach. Departure from neutrality at a given locus is assumed when the locus-specific component is needed to explain the observed pattern of diversity (alpha significantly different from 0). A positive value of alpha suggests diversifying selection, whereas negative values suggest balancing or purifying selection. Following the suggestion of Foll and Gaggiotti (2008) for minimizing the false-positive and maximizing the true-positive rate, we used the 'decisive' criterion under Jeffrey's scale of evidence to identify outlying loci.

Analysis of molecular variance (AMOVA) with Arlequin v.3.5 (Excoffier *et al.*, 2005) was used to determine the amount of genetic variability partitioned within and among populations. To analyse population differentiation, we used the F_{ST} distance method (Weir and Cockerham, 1984; Excoffier *et al.*, 1992; Weir, 1996). Molecular data consist of Euclidean distances and are unlikely to follow a normal distribution. A null distribution is therefore computed by resampling (Excoffier *et al.*, 1992). Wright's F_{ST} was also calculated for the same groups to assess the genetic differentiation of populations. Here the significance of F_{ST} was tested by comparing the observed F_{ST} with a null distribution created by 1000 random permutations of individuals among populations.

Genetic structure was also investigated through spatial analysis of molecular variance [SAMOVA 1.0 (Dupanloup *et al.*, 2002)]. SAMOVA employs a simulated procedure and uses allele frequency data – combined with the geographical coordinates of the sample populations – to identify groups of populations that have close genetic relationships. The program was run for 10,000 iterations from each of 100 random initial conditions, and tested all grouping options.

RESULTS

In total, 420 *D. subobscura* individuals from the five populations were genotyped for 11 polymorphic microsatellite markers. We found that all microsatellite loci had less then 5% missing data, so all loci were included in the analysis. The basic properties of microsatellite loci for the five populations are shown in Table 1.

Genetic diversity analysis

To determine the level of genetic diversity, we assessed a standard diversity parameter, the mean number of alleles per locus over all loci (alellic richnes), within and between populations (Table 2). The mean number of alleles per locus ranged from 10.6 to 18. The highest alellic richness per locus was detected in the Botanical Gardens population. Significant differences in allelic richness were found between the Botanical Gardens and the oakwood (Mann-Whitney U: 16.2 vs. 13.1, $Z = -2.054$, $P = 0.035$), and between the

Table 1. Basic properties of microsatellite loci

	Population						
	B	O	BG	DRG	SG	mean	S.D.
No. of gene copies	76	102	94	52	96	84	20.3
No. of loci	11	11	11	11	11	11	0.0
No. of usable loci	9	9	9	8	7	8.4	3.4
No. of polymorphic loci	9	9	9	8	7	8.4	3.4

Note: B = beechwood, O = oakwood, BG = Botanical Gardens, DRG = Derventa River Gorge, SC = Sicevo Gorge.

Table 2. Mean number of alleles per locus/all loci (allelic richness)

	Population						
Locus	B	O	BG	DRG	SG	mean	S.D.
dsub05	15	13	16	14	16	14.8	1.3
dsub04	17	16	20	17	20	18.0	1.9
dsub18	17	13	16	13	16	15.0	1.9
dsub27	20	17	19	12	16	16.8	3.1
dsub20	17	15	21	16	14	16.6	2.7
dsub01	15	13	13	11	14	13.2	1.5
dsub03	13	11	10	9	10	10.6	1.5
dsub13	15	13	10	10	12	12.0	2.1
dsub19	11	9	17	10	12	11.8	3.1
dsub02	18	12	19	10	11	14.0	4.2
dsub15	16	12	17	12	11	13.6	2.7
mean	15.8	13.1	16.2	12.2	13.8	14.2	1.7
S.D.	2.4	2.3	3.8	2.6	3.0	2.8	0.6

Note: B = beechwood, O = oakwood, BG = Botanical Gardens, DRG = Derventa River Gorge, SC = Sicevo Gorge.

Table 3. Allelic size range

	Population						
Locus	B	O	BG	DRG	SG	mean	s.d.
dsub05	29	13	17	23	18	20.6	6.2
dsub04	22	31	35	30	23	28.2	5.5
dsub18	22	12	58	15	15	24.4	19.0
dsub27	27	23	25	22	21	23.6	2.4
dsub20	23	23	24	29	18	23.4	3.9
dsub01	16	13	21	10	15	15.0	4.1
dsub03	23	12	10	9	16	5.7	2.3
dsub13	22	20	14	21	27	20.8	4.8
dsub19	18	8	25	11	18	15.6	6.6
dsub02	27	14	24	12	12	17.8	7.1
dsub15	21	18	28	16	15	19.6	5.2
mean	22.7	17.0	22.5	18.0	17.8	20.2	3.7
s.d.	3.9	6.8	12.7	7.5	4.3	7.1	3.5

Note: B = beechwood, O = oakwood, BG = Botanical Gardens, DRG = Derventa River Gorge, SC = Sicevo Gorge.

Botanical Gardens and Derventa River Gorge (Mann-Whitney U: 16.1 vs. 12.2, $Z = -2.352$, $P = 0.004$). Significant differences were also observed between the beechwood and oakwood (Mann-Whitney U: 15.8 vs. 13.1, $Z = -2.327$, $P = 0.017$), and between the beechwood and Derventa River Gorge (Mann-Whitney U: 15.8 vs. 12.2, $Z = -2.709$, $P = 0.004$).

The allelic size range for the five populations is shown in Table 3. Mean allelic size ranged between 14 and 28.2 bp. Significant differences were observed between the beechwood and oakwood (Mann-Whitney U: 22.7 vs. 17.0, $Z = -2.079$, $P = 0.003$), the beechwood and Sicevo Gorge (Mann-Whitney U: 22.7 vs. 17.8, $Z = -2.48$, $P = 0.011$), and between the Botanical Gardens and the oakwood (Mann-Whitney U: 17.0 vs. 25.5, $Z = -2.072$, $P = 0.034$).

Mean expected heterozygosity was high in all populations (0.83061 to 0.8809, Table 4). The beechwood population of *D. subobscura* had a significantly greater expected H-value than the oakwood population from the same mountain (Mann-Whitney U: 0.881 vs. 0.830, $Z = -2.101$, $P = 0.034$). A significant difference was found between the Botanical Gardens and the oakwood (Mann-Whitney U: 0.873 vs. 0.830, $Z = -2.201$, $P = 0.033$). Lower levels of expected heterozygosity were found in the Derventa River Gorge and Sicevo Gorge populations, with the lowest expected heterozygosity in the oakwood population. The molecular diversity index presumes average gene diversity over loci and the results for the five populations (beechwood, oakwood, Botanical Gardens, Derventa River Gorge, and Sicevo Gorge) were 0.875, 0.799, 0.867, 0.849, and 0.817, respectively.

The Garza-Williamson (GW) index for each population is shown in Table 5. 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 (Williamson-Natesan, 2005). All five populations had high indices (0.67 to 0.78), suggesting that none has experienced a recent bottleneck. In addition, the Wilcoxon signed-rank test was not significant for any of

Table 4. Expected heterozygosity per locus/all loci

	Population						
Locus	B	O	BG	DRG	SG	mean	s.d.
dsub05	0.9245	0.9020	0.9105	0.9148	0.9208	0.9145	0.0088
dsub04	0.9014	0.8283	0.8854	0.8605	0.9336	0.8818	0.4000
dsub18	0.8775	0.8364	0.8881	0.9005	0.9080	0.8821	0.0281
dsub27	0.9126	0.8668	0.8588	0.8688	0.8943	0.8803	0.0225
dsub20	0.8407	0.7841	0.9110	0.9118	0.8010	0.8497	0.0599
dsub01	0.8811	0.8858	0.8655	0.8831	0.8858	0.8803	0.0599
dsub03	0.8211	0.8286	0.8536	0.7086	0.7829	0.7990	0.0565
dsub13	0.8351	0.6347	0.7300	0.7101	0.6279	0.7076	0.0843
dsub19	0.8873	0.8734	0.8968	0.8848	0.9013	0.8887	0.0109
dsub02	0.9197	0.8583	0.9167	0.8702	0.8369	0.8804	0.0366
dsub15	0.8891	0.8383	0.8913	0.8129	0.8541	0.8572	0.0336
mean	0.8809	0.8306	0.8734	0.8478	0.8497	0.8595	0.0205
s.d.	0.0350	0.0726	0.0521	0.0739	0.0881	0.0643	0.0208

Note: B = beechwood, O = oakwood, BG = Botanical Gardens, DRG = Derventa River Gorge, SC = Sicevo Gorge.

Table 5. Molecular evidence for a recent bottleneck: Garza-Williamson index

	Population						
Locus	B	O	BG	DRG	SG	mean	s.d.
dsub05	0.5000	0.9286	0.8889	0.5833	0.8421	0.7486	0.1936
dsub04	0.7391	0.5000	0.5556	0.5484	0.8333	0.6353	0.1434
dsub18	0.7391	1.0000	0.2712	0.8125	1.0000	0.7646	0.2988
dsub27	0.7143	0.7083	0.7308	0.5217	0.7273	0.6805	0.0892
dsub20	0.7083	0.6250	0.8400	0.5333	0.7368	0.6887	0.1160
dsub01	0.8824	0.9286	0.5909	1.0000	0.8750	0.8554	0.1560
dsub03	0.5417	0.8462	0.9091	0.9000	0.5882	0.7570	0.1778
dsub13	0.6522	0.6191	0.6667	0.4546	0.4286	0.5642	0.1137
dsub19	0.5790	1.0000	0.6539	0.8333	0.7059	0.7544	0.1657
dsub02	0.6429	0.8000	0.7600	0.7692	0.8462	0.7637	0.0754
dsub15	0.6429	0.6316	0.5862	0.7059	0.6875	0.6677	0.0578
mean	0.6751	0.7807	0.6776	0.6966	0.7519	0.7164	0.0474
s.d.	0.1076	0.1736	0.1819	0.1793	0.1545	0.1594	0.0309

Note: B = beechwood, O = oakwood, BG = Botanical Gardens, DRG = Derventa River Gorge, SC = Sicevo Gorge.

the populations, which confirms that they did not suffer a recent bottleneck for nuclear loci (*P*-values for excess heterozygosity were 0.9262, 0.7934, 0.8398, 0.5508, and 0.1826 for the beechwood, oakwood, Botanical Gardens, Derventa River Gorge, and Sicevo Gorge population, respectively).

We found no evidence of linkage disequilibrium after we excluded linkage of microsatellite loci located on the same chromosome and using the Bonferroni correction (data not shown).

Departures from neutrality of microsatellite loci and selection

The Bayesian approach identified candidate loci under natural selection in the five natural populations tested (Table 6). None of the loci tested in the Botanical Gardens and oakwood populations showed significant departure from neutrality. Most loci in the Botanical Gardens population are under different degrees of balancing selection, and loci dsub03 (located at the U chromosome) and dsub13 (located at the E chromosome) appear to be under strong and decisive diversifying selection pressure. For the Derventa River Gorge and Sicevo Gorge populations, most microsatellite loci had different levels of purifying selection.

Genetic structure analysis

The AMOVA for all loci (Table 7) revealed that the greatest amount of genetic variance occurred within individuals ($F_{IT} = 0.03406$, $P = 0.0039$). The genetic variance among individuals within populations was 2.56% ($F_{IS} = 0.02580$, $P = 0.0097$). The amount of genetic variance among all five populations was 0.85%, and was slightly significant ($F_{ST} = 0.00848$, $P = 0.0479$). The locus-by-locus AMOVA (Table 8) confirmed the results of the overall AMOVA, and revealed that the greatest amount of genetic variance occurred within individuals ($F_{IT} = 0.10898$, $P = 0.0000$). Genetic variance among individuals within populations was 9.9% ($F_{IS} = 0.10001$, $P = 0.0000$). Genetic variance among all five populations was 0.99%, and was slightly significant ($F_{ST} = 0.00996$, $P = 0.0215$).

The results of differences in F_{ST} between populations are shown in Table 9. Differences in F_{ST} were high between the Botanical Gardens population and the beechwood ($F_{ST} = 0.00944$, $P = 0.000$), Derventa River Gorge ($F_{ST} = 0.01340$, $P = 0.000$), and Sicevo Gorge ($F_{ST} = 0.01971$, $P = 0.000$) populations. There was also a significant difference in F_{ST} between the beechwood and Derventa River Gorge populations ($F_{ST} = 0.00885$, $P = 0.040$).

The SAMOVA, which defined the genetic structure of populations by simulated annealing, revealed genetic differentiation among groups. The highest differentiation was among three groups of populations (beechwood + oakwood, Botanical Gardens only, Derventa River Gorge + Sicevo Gorge; $F_{ST} = 0.62$, $P < 0.0008$).

DISCUSSION

Under a scenario of rapid climate changes, glacial refugia are expected to provide stable conditions for long-term persistence in biodiversity (Stewart *et al.*, 2010). The importance of refugia is increasingly being recognized (Noss, 2001; Bennett and Provan, 2008). Although the term 'refugia' was originally used to refer to locations where species survived the last glaciation (Bennett and Provan, 2008), it is now increasingly used to refer to areas that should be conserved in the hope of limiting impacts of rising global temperatures in the twenty-first century (Barnosky, 2008; Rull, 2009). In the present study, we wished to identify glacial refugia within the central Balkans, by identifying specific patterns that could be evaluated by molecular markers of microsatellite DNA. Within this framework, we expected conspecific populations to be geographically structured and to have the highest genetic diversity in known glacial refugia (i.e. within the areas of continuous persistence of populations). According to our results, the pattern of microsatellite variability of the five populations studied is in line with that of five European populations (Pascual *et al.*, 2001). For instance, the

Table 6. Bayesian assessment of loci under selection (α is a locus-specific effect; *P* is posterior probability that locus is an outlier)

	B			O			BG			DRG			SG			Overall		
Locus	α	*P*	F_{ST}	α	*P*	F_{ST}	α	*P*	F_{ST}	α	*P*	F_{ST}	α	*P*	F_{ST}	α	*P*	F_{ST}
dsub05	−0.04	0.08	0.03	−0.02	0.35	0.08	−0.16	0.34	0.04	**−0.51**	0.50	0.05	**−0.88**	0.73	0.03	−0.02	0.06	0.02
dsub04	0.01	0.06	0.05	0.08	0.28	0.10	−0.04	0.31	0.05	**−0.52**	0.50	0.05	**−0.79**	0.74	0.04	−0.03	0.08	0.02
dsub18	−0.04	0.08	0.04	−0.27	0.30	0.10	0.16	0.34	0.05	**−0.50**	0.50	0.06	**−0.64**	0.54	0.04	−0.36	0.45	0.01
dsub27	−0.07	0.10	0.03	0.06	0.33	0.08	0.22	0.33	0.06	0.33	0.38	0.1	0.03	0.30	0.07	−0.01	0.04	0.02
dsub20	0.00	0.07	0.05	−0.35	0.29	0.10	−0.40	0.39	0.03	**−0.65**	0.55	0.05	**0.51**	0.55	0.10	−0.01	0.04	0.02
dsub01	0.02	0.08	0.04	0.13	0.09	0.08	0.37	0.40	0.06	−0.03	0.35	0.08	−0.33	0.39	0.06	**−1.23**	0.97	0.01
dsub03	0.00	0.08	0.07	0.45	0.33	0.11	**0.91**	0.81	0.11	**0.71**	0.66	0.14	**0.86**	0.81	0.14	−0.34	0.36	0.01
dsub13	0.01	0.06	0.06	0.19	0.45	0.14	**1.31**	1.00	0.15	**0.77**	0.75	0.14	**0.85**	0.88	0.14	−0.01	0.04	0.02
dsub19	0.00	0.08	0.05	0.27	0.36	0.12	**−1.15**	0.85	0.02	0.07	0.36	0.08	**−0.54**	0.58	0.05	−0.02	0.04	0.02
dsub02	0.00	0.08	0.03	−0.17	0.34	0.12	−0.41	0.42	0.03	**0.52**	0.55	0.11	**0.80**	0.81	0.14	−0.02	0.05	0.02
dsub15	−0.08	0.10	0.03	−0.17	0.33	0.09	**−0.70**	0.57	0.03	−0.10	0.34	0.07	0.20	0.35	0.08	**−1.47**	1.00	0.00

Jeffrey's interpretation: 050–0.76, barely worth mentioning; 0.76–0.91, substantial; 0.91–0.97, very strong; 0.99–1.0, decisive.
Loci marked with bold alpha values are the ones the Bayesian approach suggested are under selection.

Note: B = beechwood, O = oakwood, BG = Botanical Gardens, DRG = Derventa River Gorge, SC = Sicevo Gorge.

Table 7. AMOVA results for all loci

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indices	*P*-value
Among populations	4	19.414	0.02392 Va	0.85	$F_{ST} = 0.00848$	0.04790*
Among individuals within populations	205	587.952	0.07212 Vb	2.56	$F_{IS} = 0.02580$	0.00978**
Within individuals	210	572.000	2.72381 Vc	96.59	$F_{IT} = 0.03406$	0.00391**
Total	419	1179.367	2.81985			

Note: Significance tests 1023 permutations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 8. Locus by locus AMOVA results (as a weighted average over loci)

Source of variation	Sum of squares	Variance components	Percentage of variation	Fixation indices	*P*-value
Among populations	35.216	0.04748	0.99619	$F_{ST} = 0.00996$	0.02151*
Among individuals within populations	951.846	0.47189	9.90140	$F_{IS} = 0.10001$	0.00000***
Within individuals	806.000	4.24653	89.10241	$F_{IT} = 0.10898$	0.00000**
Total	1793.062	4.765			

Note: Significance tests 1023 permutations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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

	Population									
	B		O		BG		DRG		SG	
	F_{ST}	*P*	F_{ST}	*P*	F_{ST}	*P*	F_{ST}	*P*	F_{ST}	*P*
B										
O	0.00453	0.06								
BG	0.00944	0.0***	0.00401	0.05						
DRG	0.00885	0.04*	0.00671	0.05	0.01340	0.00***				
SG	0.01052	0.00***	0.00383	0.06	0.01971	0.00***	0.00564	0.09		

Note: Significance tests 1023 permutations. B = beechwood, O = oakwood, BG = Botanical Gardens, DRG = Derventa River Gorge, SC = Sicevo Gorge. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

expected heterozygosity for our localities is between 0.831 and 0.881, whereas the mean expected heterozygosity for the five European populations is 0.893. If we take into account standard diversity indices (allelic richness per locus, allelic size range), the variability detected in the Balkan populations is lower than that for Europe. The allelic richness per locus ranged from 12.2 to 16.2, which is similar to the allelic richness per locus (13.3–16.3) for other European populations (Pascual *et al.*, 2001). The results for microsatellite variability of

the present five populations generally fit into the European distribution; and the Balkan Peninsula was probably not a glacial refugium for *Drosophila subobscura*, in particular. However, more extensive research is required to evaluate that conclusion.

The beechwood and Botanical Gardens populations fit the European pattern of microsatellite variability (Pascual *et al.*, 2001), but lower expected heterozygosity, allelic richness per locus, and allelic size range were found in two populations collected from very specific habitats (Derventa River Gorge and Sicevo Gorge). The habitats at both the latter sites are ecologically unusual. Canyons and gorges in the southern and central part of the Balkan Peninsula show great floristic diversity (Grebenscikov, 1950; Jovanovic and Jovanovic-Dunjic, 1986; Lakusic and Redzic, 1989). 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 disfavour the heterozygote advantage.

Microsatellite differentiation among the five populations in the present study was weak but significant. This is consistent with reports for other European populations (Pascual *et al.*, 2001). This result is important, given the known high migration rate for *Drosophila subobscura* (Taylor *et al.*, 1984). The five populations in the present study were collected from very distinct and specific habitats. Two populations are in the same mountainous area, but in very distinct microhabitats. Another two populations are in highly ecologically stable gorge ecosystems. The fifth (Botanical Gardens) has a very specific microclimate and experiences high anthropogenic activity. Our results suggest that populations in different habitats should be subjected to habitat-specific selection regimes. The populations appear structured according to microhabitat characteristics (i.e. their adaptation to a particular microhabitat). Pairwise F_{ST} among populations suggests that the Botanical Gardens population is significantly different from the other ones. The results of SAMOVA confirmed that the greatest differentiation among the populations is observed when analysed as three groups: (1) the beechwood + oakwood populations, (2) the Botanical Gardens population on its own, and (3) the Derventa River Gorge + Sicevo Gorge populations.

Bowers *et al.* (1973) and Greenbaum *et al.* (1978) attribute the genetic similarity of various populations of *Peromyscus maniculatus* and *P. melanotis* to their small population sizes. In other words, the genetic similarity of those populations could be a result of population isolation in their local habitats (instead of high gene flow). These data also argue against the assumption that monomorphism in isolated populations arose by drift and instead support a model of centrifugal speciation. In contrast, our results for *D. subobscura* 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). Furthermore, *D. subobscura* appears to adapt quickly. It colonized South and North America around the late 1970s (Bechenbach and Prevosti, 1986; Ayala *et al.*, 1989), where it spread rapidly and successfully (Prevosti *et al.*, 1989; Pascual *et al.*, 1993). It has very rapidly adapted to its new environments and, as a consequence, has developed clines for some chromosomal arrangements (Prevosti *et al.*, 1988) and for wing size (Huey *et al.*, 2000; Gilchrist *et al.*, 2001, 2004), similar to those found in Old World populations.

Our results show no significant departures from neutrality for all the loci tested in the beechwood and oakwood populations. In the Derventa River Gorge and Sicevo Gorge

populations, however, most microsatellite loci are under different levels of purifying selection (selective removal of deleterious alleles). This could explain the lower level of expected heterozygosity in these populations. The same microsatellite loci originating from two populations (Derventa River Gorge and Sicevo Gorge) showed the same selection response. We expected such a result, because these two localities are ecologically similar, and both have all the characteristics of ecologically stable habitats. Most of the analysed loci from the Botanical Gardens population are under different degrees of balancing selection: loci dsub03 (at the U chromosome) and dsub13 (at the E chromosome) are under strong and decisive diversifying selection pressure. This is to be expected, as this particular population came from a central, urban part of Belgrade, which is no doubt influenced by anthropogenic activity. This specific microhabitat 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.

Our results suggest that the microsatellite variation in the populations under study is ecologically significant. Even though microsatellite loci are theoretically considered to be selectivity neutral, selection may influence the variability of a microsatellite locus in two ways: (1) a microsatellite itself may have an important function in the genome, and selection maintains this function by constraining microsatellite variability; (2) alternatively, selection may not act on the microsatellite itself but on a DNA region closely linked to the microsatellite. Under this scenario, the microsatellite might be influenced by selective processes (e.g. 'background selection' or 'selective sweep') 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. Thus, we believe that the position along the chromosome, where the microsatellite is located, determines its variability, with microsatellites located in low-recombining regions.

Background selection purges deleterious mutations and thus reduces the level of linked, neutral variation. Therefore, a positive correlation is expected between the recombination rate and neutral polymorphism. Selective sweep and hitchhiking occur when a selectively favoured mutation occurs in a population and is subsequently fixed. In that case, the frequencies of polymorphisms at linked loci will be altered (Maynard Smith and Haigh, 1974). The size of the flanking neutral region affected by such a selective sweep depends on the recombination rate and the selection intensity of the favourable mutation. In this model, a correlation between the recombination rate and polymorphism is explained by recurrent fixation of new advantageous mutations. Background selection and hitchhiking were originally considered to explain the observed correlation between rates of recombination and level of nucleotide polymorphism in *D. melanogaster* (Begun and Aquadro, 1992; Aquadro *et al.*, 1994). However, the same mechanisms are, in principle, expected to affect levels of microsatellite polymorphisms, even though the predictions under both models are slightly different for genomic regions with higher mutation rates, such as microsatellites (Schug *et al.*, 1998; Wiehe, 1998; Schlötterer and Wiehe, 1999).

The adaptive population divergence seen in our populations of *D. subobscura* indicate that local adaptations at the molecular level (probably between chromosomal inversions and microsatellite loci) can occur despite high gene flow and large effective sizes (Pascual *et al.*, 2001). Furthermore, our results highlight the contribution of the analysis of microsatellite loci to demonstrating local adaptation.

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