UDC 575.
https://doi.org/10.2298/GENSR1901213E
Original scientific paper

NUCLEOTIDE DIVERSITY OF *Cyt b* GENE IN *Drosophila subobscura* Collin

Pavle ERIĆ[1*], Mihailo JELIĆ[2], Marija SAVIĆ VESELINOVIĆ[2], Bojan KENIG[1], Marko ANĐELKOVIĆ[1,2,3], Marina STAMENKOVIĆ-RADAK[1,2]

[1] Institute for Biological Research "Siniša Stanković", University of Belgrade, Belgrade, Serbia
[2] Faculty of Biology, University of Belgrade, Belgrade
[3] Serbian Academy of Sciences and Arts, Belgrade

Erić P., M. Jelić, M. Savić Veselinović, B. Kenig, M. Anđelković, M. Stamenković-Radak(2019): *Nucleotide diversity of Cyt b gene in Drosophila subobscura* Collin.-Genetika, Vol 51, No.1, 213-226.

Mitochondrial DNA variability of *Drosophila subobscura* Collin from Southeastern Serbia was studied with respect to Restriction Site Analysis (RSA) of complete mitochondrial genome and the nucleotide sequence of Cytochrome b (*Cyt b*) gene. The aim was to shed more light on the evolutionary forces that shape mtDNA variation of this species. Samples were collected from two sites in the foothills of the Balkan Mountains. No genetic differentiation was found between groups and most of the variation was observed within them. Restriction analysis revealed two main haplotypes and several rare ones. The sequencing of *Cyt b* gene showed larger number of haplotypes, among which, one is being the most common. The majority of singletons differed from the most frequent haplotype by one nucleotide change. Although some of the observed nucleotide differences may affect their host's fitness, the observed pattern of variation is consistent with the seasonal fluctuations in population size.

Keywords: mtDNA, haplotype network, singleton, fruit fly, population history

INTRODUCTION

The Palearctic fruit fly *Drosophila subobscura* Collin is characterized by homogenous geographic pattern of mitochondrial DNA (mtDNA) variation. Across the species range there is high prevalence of two almost equally frequent haplotypes (named I and II) that exist together

Corresponding author: Pavle Erić, Institute for Biological Research "Siniša Stanković", University of Belgrade, Despot Stefan blvd. 142, 11000 Belgrade, Serbia, pavle.eric@ibiss.bg.ac.rs

with the population specific rare ones (AFONSO *et al.*, 1990; CASTRO *et al.*, 1999; STAMENKOVIĆ-RADAK *et al.*, 2012). Due to this pattern, *D. subobscura* has been proven as a good model species for studying evolutionary forces that shape and maintain sympatric mtDNA variation (CASTRO *et al.*, 2010; KURBALIJA NOVIČIĆ *et al.*, 2015; JELIĆ *et al.*, 2015). Up to now, great body of evidence shows that both adaptively neutral and selective forces shape mtDNA variation of this species. The pattern of nucleotide variation with excess of singletons reveals mutational events during population expansion (CASTRO *et al.*, 2010; CHRISTIE *et al*., 2010). Also, laboratory studies have shown the effect of mtDNA upon host's fitness. One of such study compared several sympatric haplotypes upon uniform nuclear genetic background (CHRISTIE *et al*., 2011). While many of the rare haplotypes show similar fitness to haplotypes I and II, some of them seem to have a negative effect upon their host, implying that negative selection also acts in natural populations.

The differences in life history traits and mating behavior between the two most frequent haplotypes exist only when they are compared based on their own nuclear genetic background (CASTRO *et al.*, 2003; CHRISTIE *et al.*, 2004), and disappear if compared on the uniform nuclear genetic background (CHRISTIE *et al.*, 2011). In natural populations, there is a trend of seasonal haplotype change; while haplotype II is generally more common throughout the year, haplotype I is more frequent in spring (CHRISTIE *et al.*, 2010). This pattern indicates adaptive differences in the wild populations. Almost equal presence of the two haplotypes across the species range suggests the action of some form of balancing selection, with negative frequency dependent selection being most likely (KURBALIJA NOVIČIĆ *et al.*, 2015; ARNQVIST *et al.*, 2016).

In the present paper we further analyze nucleotide variation of mtDNA in natural populations of *D. subobscura* from Southeastern Serbia. Specifically, we complement Restriction Site Analysis (RSA) of the entire mtDNA with the analysis of nucleotide variation in *Cyt b* gene, and discuss evolutionary forces responsible for the genetic pattern observed in nature.

MATERIALS AND METHODS

Samples

D. subobscura individuals were collected in June 2015 in Southeastern Serbia in the foothills of Stara Planina (the Balkan Mountains) (N43º25' E22º25'). Specimens were sampled from two sites approximately 3 km apart: Stara Kalna (SK) and Gabrovnica (G). Isofemale lines (IFL) were established, each from a single gravid female (30 from SK, 49 from G). All IFL were maintained under constant laboratory conditions, at 19ºC, ~60% relative humidity, light of 300 lx, and photoperiod of 12 h light and 12 h dark. The F_1 progeny of IFL was used to determine maternal mitochondrial haplotypes by means of Restriction Site Analysis (RSA) and sequencing of *Cyt b* gene.

mtDNA Restriction Site Analysis (RSA)

A method described by MARTINEZ *et al.* (1992) was used to obtain an enriched fraction of mtDNA. The obtained mtDNA was digested with five restriction enzymes (*Eco*RI, *Eco*RV, *Hind*III, *Hae*III, and *Hpa*II), selected for their ability to detect mtDNA polymorphisms (AFONSO *et al.*, 1990; CASTRO *et al.*, 1999; JELIĆ *et al.*, 2012). FastDigest restriction enzymes were used (ThermoScientific, Waltham, Massachusetts, USA) according to the manufacturer's recommendations. The mtDNA was digested with each enzyme separately. Digested DNA fragments were separated on horizontal 0.8-1.2% agarose gels with ethidium bromide in final concentration of 0.1 μg/mL. λ DNA digested with *Hind*III and λ DNA double digested with

*Hind*III-*Eco*RI were used as size standards. After electrophoresis, gels were photographed using a Bio-Rad Gel Doc 1000 (Bio-Rad Laboratories, Hercules, California, USA). When rare restriction patterns appeared, double digestions were used to determine the exact location of the restriction sites. The restriction patterns obtained, using a given enzyme and the haplotypes were named according to the notation of LATORRE *et al.,* (1992), CASTRO *et al*., (1999) and STAMENKOVIĆ-RADAK *et al.,* (2012). RSA analysis was conducted on the entire set of IFL.

Sequencing of Cyt b gene

A 893-bp fragment corresponding to *Cyt b* gene of mtDNA was PCR amplified and sequenced from the mtDNA of the randomly chosen set of 41 IFL (20 from SK and 21 from G). Previous RSA analyses (AFONSO *et al.*, 1990; OLIVER *et al*., 2002; CHRISTIE *et al.*, 2010; CASTRO *et al.,* 2010) showed abundance of restriction sites in this gene. For PCR and sequencing reaction *Cyt b*-F 5'-TTAT GGTT GATT ATTA CGAA-3' and *Cyt b*-R 5'- CAAA ACAT ATGC TTAT TCAA-3' primers were used (GAO *et al.,* 2007). The PCR cycling conditions consisted of an initial denaturation step at 94ºC for 3 min, 35 cycles: at 94ºC for 50 s, 51.5ºC for 1 min and 72ºC for 1 min; with a final extension at 72ºC for 3 min. PCR products were purified using the QIAquick PCR Purification kit (QIAGEN, Hilden, Germany). Sequencing reactions were performed with both primers (Macrogen inc. Amsterdam, The Netherlands). The sequences were aligned using the software BioEdit v.7.2.5 (HALL, 1999).

Presence of Wolbachia

To exclude cytoplasmic incompatibility promoted by the presence of *Wolbachia*, a PCR test using 16S-6 primer set was conducted according to SIMÕES *et al.,* (2011). This primer pair is highly sensitive in detecting majority of supergroups of *Wolbachia*, and gives no falsely positive results. Test was conducted on total genomic DNA for each IFL. During mtDNA extraction, before the final step of alkaline lysis, 15 μL of the total genomic DNA was saved for the purpose of this test. Two different *Drosophila* samples containing *Wolbachia* were used as positive controls: *D. tristis* captured from SK, and *D. melanogaster* stock no. 5 (Bloomington Stock Centre).

Statistical analysis

Nucleotide and haplotype diversity was calculated. Tajima's D (TAJIMA, 1989), Fu's Fs (FU, 1997), Fu and Li's D-F (FU and LI, 1993) and Fay and Wu's H (FAY and WU, 2000) tests were used to test departure from neutrality for the mtDNA haplotype distribution in populations. McDonald-Kreitman test (MCDONALD and KREITMAN, 1991) was implemented to compare ratio of non-synonymous to synonymous change within and between species. Changes in population size were examined by calculating the observed and expected pairwise differences (mismatch distribution) (ROGERS and HARPENDING, 1992). Tests were conducted for SK and G separately, and for the total sample set. The analysis of molecular variance (AMOVA) was implemented in order to partition variation between and within two samples. The above parameters and tests were conducted using DNASP v.5.0 (LIBRADO and ROZAS, 2009) and Arlequin v.3.5.1.2 (EXCOFFIER and LISCHER, 2010). Estimation of gene genealogies from *Cyt b* gene was obtained using PopART software which was then used to construct TCS phylogenetic network (LEIGH and BRAYANT, 2015); sequences were collapsed into haplotypes based on statistical parsimony

(CLEMENT *et al.*, 2002). *D. madeirensis* (GenBank: EF216274.1) sequence from the NCBI site (*www.ncbi.nlm.nih.gov*) was used as an out-group.

RESULTS

Each of the five enzymes gave two alternative restriction patterns (Figure 1), which formed six haplotypes, in total. Patterns which were not priory observed and published are designated by the next available alphabet letter. All recorded haplotypes were present in SK, while only three of those are found in G. In both samples the prevalence of haplotypes I and II was recorded. They were of equal frequency in SK, while haplotype I was more frequent in G. Haplotype frequencies and haplotype diversity are presented in Table 1. One (VI) and three (III, IV and V) rare haplotypes are derived from haplotypes I and II, respectively. All rare haplotypes differ by one restriction site change compared to the haplotypes I and II. The network connecting the six haplotypes is shown in Figure 2. The Tajima's D was negative in SK, positive in G, and negative if total sample is considered, but none was statistically significant (Table 1). The AMOVA shows that there is little variation between flies from two localities (FST=-0.01518, p= 0.58358), while the majority of variation exists within samples.

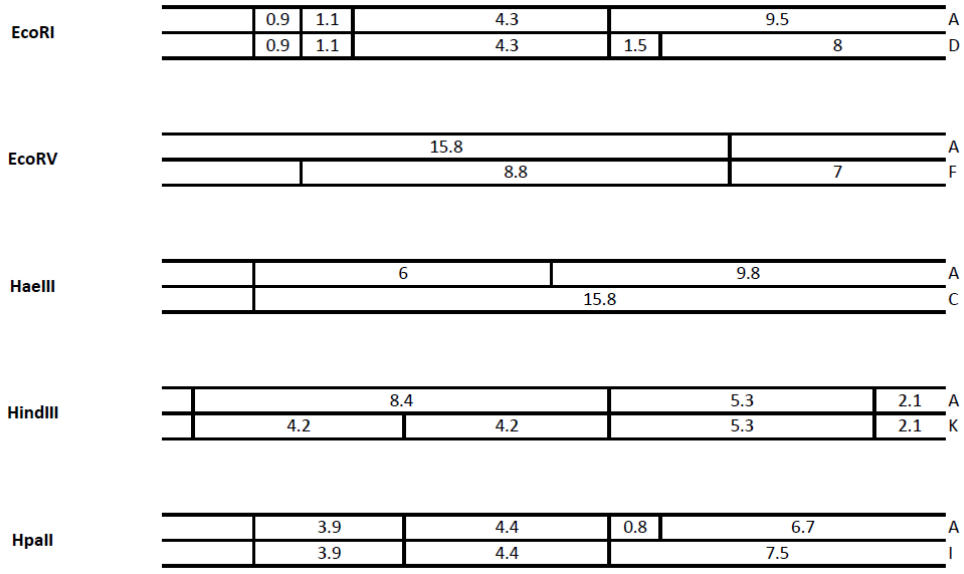

Figure 1. Scheme of the restriction patterns of *D. subobscura* mtDNA obtained with five restriction enzymes. Fragment sizes are indicated in kilobase pairs. Capital letters denote restriction patterns.

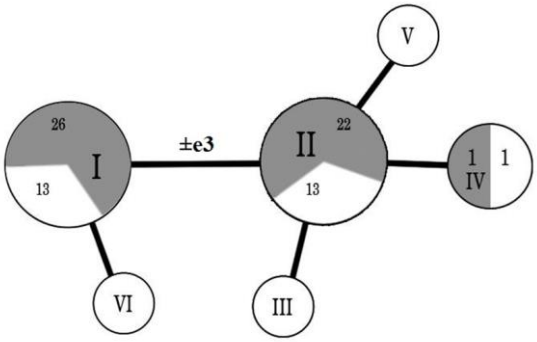

Figure 2. Network of six haplotypes of the SK (depicted in white) and G (depicted in gray) samples of *D. subobscura* obtained by RSA. The haplotypes are connected in a way that minimizes the total number of site changes. Number within circles corresponds to the number of IFL with the specific haplotype. *e3* restriction site of *Hae*III makes difference between the two main haplotypes.

Table 1. Percentage of D. subobscura IFL with different haplotypes, restriction patterns of the haplotypes, parameters of diversity and Tajima's D test

	Haplotype Frequency (%)			Restriction patterns				
Haplotypes	SK	G	Total	*Eco*RI	*Eco*RV	*Hae*III	*Hind*III	*Hpa*II
I	43.33	53.06	49.37	A	A	A	A	A
II	43.33	44.90	44.30	A	A	C	A	A
III	3.33	/	1.27	D	A	C	A	A
IV	3.33	2.04	2.53	A	A	C	A	I
V	3.33	/	1.27	A	F	C	A	A
VI	3.33	/	1.27	A	A	A	K	A
no. of IFL	30	49	79					
haplotype	0.6414	0.5272	0.566					
diversity	±0,054	±0,026	±0,027					
Tajima's D	-1.039	0.403	-0.832					

The analysis of 893-bp mtDNA fragment of the *Cyt b* gene showed presence of 14 haplotypes, one very frequent, C1, and 13 singletons (Table 2). In total 16 polymorphic sites were found in 41 individuals sequenced. Ten and six nucleotide changes were observed in G and SK, respectively. The network connecting haplotypes is shown in Figure 3. Majority of rare haplotypes are connected with the most frequent one with only one mutational change. The exceptions are haplotypes C8 and C5 with two and three mutational steps, respectively. The out-

group species *D. madeirensis* is separated from *D. subobscura* cluster by 27 mutational steps. With respect to the polymorphic restriction sites, site 446 corresponds to the *Eco*RV target that distinguishes haplotype II from haplotype V by a T-C change. Rare haplotypes III and V also have additional substitutions in *Cyt b* gene. Twelve substitutions are synonymous, and all non-synonymous substitutions are from location G (Table 3). Table 4. shows nucleotide and haplotype diversity of *Cyt b* gene.

Table 2. Nucleotide sequences of the D. subobscura Cyt b gene. Seq., sequence; T., Total; D.m, D. madeirensis; s, synonymous change; n, non-synonymous change

Seq.	Polymorphic sites																															SK	G	T
						1	1	1	2	2	2	2	2	2	3	3	3	3	3	4	4	4	5	5	5	5	6	6	7	7	7	8	8			
	1	2	4	6	9	0	5	5	0	1	2	3	4	7	7	0	1	2	3	5	6	4	4	5	1	5	8	9	2	6	1	2	4	8	0	4			
	4	0	1	2	2	2	2	5	9	8	4	1	2	2	5	5	4	0	2	9	2	0	6	9	5	1	7	3	9	0	5	5	4	1	7	2			
	s	s	s	s	s	n	s	s	s	s	s	n	s	s	s	s	s	s	s	s	s	s	s	n	s	s	s	s	s	n	s	s	n	s	s			
C1	T	A	C	G	C	G	T	A	G	C	T	G	C	T	T	C	G	C	T	G	C	G	T	G	T	T	C	T	A	T	C	G	C	G	T	T	16	12	28
C2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	1		1
C3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1		1
C4	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1		1
C5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1		1
C6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.		1	1
C7	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1	1
C8	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.		1	1
C9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.		1	1
C10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.		1	1
C11	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1	1
C12	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1	1
C13	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1	1
C14	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1	1
D.m	C	G	T	A	T	.	.	G	A	A	C	A	A	.	C	T	A	T	.	A	T	A	.	A	C	C	T	C	.	C	.	A	T	.	C	C			
T																																					20	21	41

Table 3. Non-synonymous substitutions in the D. subobscura Cyt b gene

Haplotype	Polymorphic sites					SK	G	Total
			1	2	2			
	3	7	5	3	6			
	4	7	3	8	0			
C1	V	V	V	P	R	20	18	38
C6	.	.	.	.	Q	/	1	1
C8	M	.	I	.	.	/	1	1
C9	.	.	.	L	.	/	1	1
D. madeirensis	.	M	I	.	.			
no. of IFL						20	21	41

V, P, R, Q, M, I, L correspond to valine, proline, arginine, glutamine, methionine, isoleucine and leucine, respectively

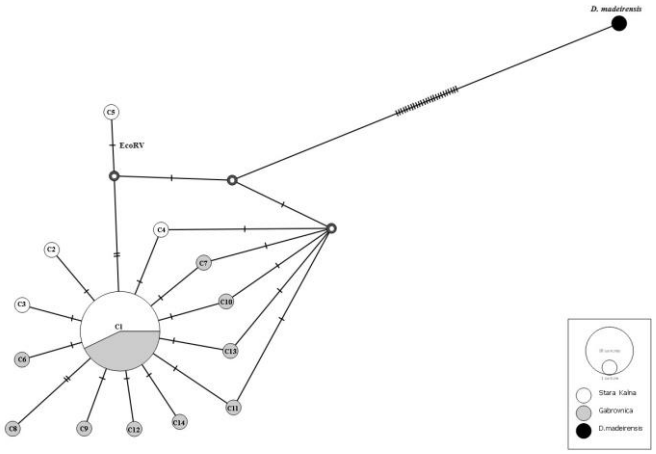

Figure 3. Network of 14 *D. subobscura Cyt b* haplotypes, with *D. madeirensis* as an out-group. One nucleotide that coincides with the polymorphic restriction site of *Eco*RV is indicated. The size of the circles correspond to the number of the IFL with the specific haplotype. Origin of IFL is indicated in white (SK) and gray (G).

Table 4. Nucleotide and haplotype diversity of D. subobscura in the analyzed fragment of Cyt b gene

	Nucleotide diversity		Haplotype diversity	
sample location	π	SD	Hd	SD
SK	0.00067	0.00032	0.368	0.135
G	0.00107	0.00026	0.686	0.115
Total	0.00087	0.00022	0.539	0.096

The Tajima, Fu, Fu and Li, and Fay and Wu's tests gave negative values (Table 5). The Tajima's D, Fu's Fs, and Fay and Wu's H values were statistically significant for both samples, and for total sample; Fu and LI's D values were not significant in any case, and F was statistically significant only for the whole sample set. Generally, the highest significantly negative values are observed in the total sample, while G always had higher absolute values and lower p values compared to SK. The results of McDonald-Kreitman test are presented in Table 6. Due to the absence of non-synonymous changes in SK, the observed neutrality index was zero, and G test not applicable. In G and in total sample, neutrality index was positive and above 1. Significance was observed only for G, but not for the total sample. Our results show that the ratio of non-silent to silent variation is greater within species than between species.

Table 5. Results of Tajima, Fu, Fu & Li and Fay & Wu tests for the analyzed region of Cyt b gene in D. subobscura (for Fu & Li and Fay & Wu tests D. madeirensis served as an out-group)

Test:	Tajima		Fu		Fu & Li				Fay & Wu	
sample location	D	p	Fs	p	D	p	F	p	H	p
SK	-2.056	**	-2.473	**	-1.014		-1.563		-5.116	**
G	-2.269	***	-8.709	***	-1.220		-1.848		-8.595	***
Total	-2.534	***	-15.359	***	-1.944		-2.583	*	-14.839	***

*, p<0.05; **, p<0.02; ***, p<0.001

Table 6. Results of McDonald-Kreitman test between D. subobscura sequences and D. madeirensis for the analyzed region of Cyt b gene

Sample location	N	F, p	G (W)	p	G (Y)	p
SK	0	1	na	na	na	na
G	14.667	0.021413*	5.661	0.01735*	4.033	0.04463*
Total	6.333	0.149266	2.787	0.09505	1.546	0.21371

N, neutrality index; F, p, Fisher's exact test P-value (two tailed); G (W), G(Y), G test with Williams' and Yates' correction respectively; na, not applicable, * P<0.05

The distribution of the observed and expected pairwise differences in the total sample and two locations separately is presented in Figure 4. In the total sample set, there is a perfect correspondence to the model of population expansion. If the two samples are observed separately the fit is not so accurate. SK is characterized with the slight bimodality. The additional small peak is observed at the position of three pairwise differences and is the result of the presence of sequence C5 which is connected to the most frequent haplotype C1 by three nucleotide changes. G shows unimodal mismatch distribution, but the fit is not as accurate as for the total sample which is a consequence of the excess of singletons that differ from C1 by one nucleotide change.

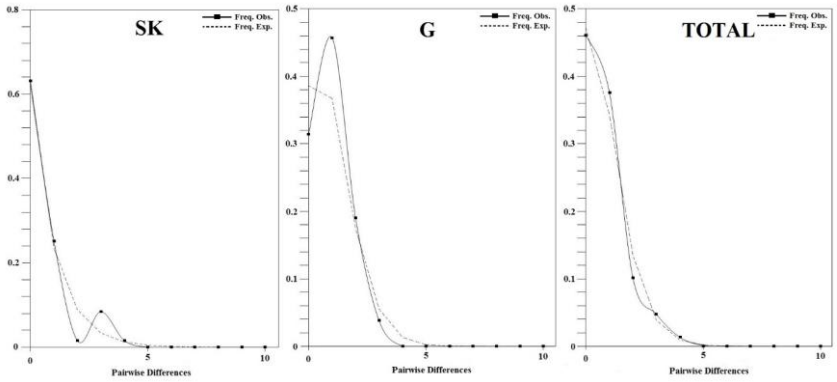

Figure 4. Fequencies of the observed and expected pairwise differences in the sample. Exp, expected values; Obs, observed values

As for the RSA analysis, the AMOVA with *Cyt b* sequence also showed that the majority of variation exists within the two sample sites, with little variation between them (F_{ST}=-0.00055, p= 0.84555).

All IFL were negative for the presence of *Wolbachia*. Other signs of cytoplasmic incompatibility, such as distortions in sex ratio, or embryo mortality in crosses between strains were not observed.

DISCUSSION

This study describes the variability of mtDNA in *D. subobscura* implementing two different methods. The RSA gives overview of the variability of the entire mitochondrial genome, while the analysis of the *Cyt b* gene provides information for just a fragment of it. RSA can be considered quite robust technique since it is limited only to the restriction site sequences and cannot provide data on the exact nucleotide changes in the polymorphic restriction sites. However, this approach was able to make a difference between the two haplogroups of *D. subobscura* (haplotype I and its derivatives from haplotype II and its derivatives). On the other hand, sequencing of just one fragment of mtDNA, such as *Cyt b* gene, provides genetic data with higher resolution: the exact nucleotide changes are defined and, in the case of non-synonymous substitutions, amino acid replacements. Regardless the different scale and power of the two approaches, the information they provided in this study of mtDNA variability in *D. subobscura* is highly concordant. Both show excess of singletons with prevalence of single stepwise changes from one (*Cyt b*) or two (RSA) main haplotypes.

The haplotype frequency spectrum obtained by RSA is in the agreement to the earlier studies conducted across the species range (AFONSO *et al.*, 1990; LATORRE *et al.*, 1992; CASTRO *et al.*, 1999; STAMENKOVIĆ-RADAK *et al.*, 2012) considering the prevalence of the haplotypes I and II, and joint frequency of the rare haplotypes. Samples taken from the two sites did not show genetic differentiation and shared one rare haplotype, which may be the result of their proximity

and high rate of gene flow observed in this species (AYALA *et al*., 1989). Haplotype diversity (0.566 for the total sample set) resembles the other populations from the central part of the Balkan Peninsula where values between 0.436 and 0.559 have been observed with the same set of restriction endonucleases (STAMENKOVIĆ-RADAK *et al.*, 2012).

Prior to the results of sequencing of *Cyt b* gene reported here, another population genetic study of the protein coding mitochondrial *ND5* gene has been conducted in *D. subobscura* populations originating from southwestern part of population range, namely the Balearic Islands, and the Iberian Peninsula (CASTRO *et al.*, 2010). Apart from the two prevalent haplotypes of the *ND5* gene in contrast to only one in *Cyt b*, the general pattern of nucleotide diversity is quite similar: there is an excess of singletons that are different from the main haplotype mostly by one nucleotide change. Nucleotide diversity of *Cyt b* gene (range 0.00067-0.00107; 0.00087 for the total sample) is of the similar magnitude compared to the *ND5* gene (range 0.00092-0.00266; 0.00221 for the total sample), however lower and more similar in value to the continental, Iberian population of La Canyada. BALLARD and KREITMAN (1994) have analyzed nucleotide diversity in *Cyt b* gene in tree species of *Drosophila: D. melanogaster* (0.0009), *D. simulans* (0.0003) and *D. yakuba* (0.0014). Values observed for *D. subobscura* are of similar magnitude as those recorded for the above mentioned species.

The observed excess of singletons both in RSA and in *Cyt b* is responsible for the negative values of parameters that measure departure of haplotype distribution from neutrality. The power of RSA to detect polymorphism was lower giving rise to non-significant departure of Tajima's D from zero, compared to the *Cyt b* analysis. Nevertheless the Tajima's D was generally negative, as also the indices of the Fu's, Fu and Li's and Fay and Wu's tests for *Cyt b* sequences.

As for the McDonald-Kreitman test, positive values of neutrality indices in G and in total sample indicate an excess of non-silent polymorphism compared to divergence. Non-synonymous substitutions are exclusively present in G where significant departure from neutrality is observed. The analysis of different mtDNA gene in mice and humans generally gave excess of amino acid polymorphism, relative to divergence (NACHMAN *et al.*, 1994; NACHMAN *et al.*, 1996; TEMPLETON, 1996). The same is observed in *Drosophila* (KANEKO *et al*., 1993; BALLARD and KREITMAN, RAND *et al.*, 1994), especially for *Cyt b* gene (BALLARD and KREITMAN, 1994).

Two mutually nonexclusive scenarios could have caused the observed pattern of nucleotide variation and the statistical results that measure departure from the neutral evolution model. The first is purifying selection that has driven the most frequent haplotype close to fixation. The second is population expansion after the population has gone through a bottleneck. An array of nucleotide substitutions has been observed in *Cyt b* in this work. As in the work of CHRISTIE *et al.*, (2011) some of them could have possibly impaired their host's fitness. The results of McDonald-Kreitman test is in agreement with this notion especially for sequences sampled from site G. RAND and KANN (1996; 1998) have interpreted this excess of amino acid replacement in light of nearly neutral model (OHTA, 1992a; 1992b) that predicts accumulation of mildly deleterious alleles that persist for short time within a population and do not contribute to divergence (NACHMAN, 1998; RAND and KANN, 1998; WEINREICH and RAND, 2000; MEIKLEJOHN *et al.*, 2007). Also, the Fay and Wu's test which is less sensitive in detecting population expansion gave significant values implying that some of the observed singletons indeed

compromise fitness in natural populations. However, the results of the observed and expected pairwise differences indicate that the evolution of a population as a whole is largely influenced by changes in population size. EYRE-WALKER (2002) showed that recent increase in effective population size can generate artifactual evidence of positive selection if substitutions are slightly deleterious and if there is no selection upon synonymous codon use.

CASTRO *et al.,* (2010) have discussed the time scale of the past non-adaptive evolutionary events that could have influenced mtDNA variation in *D. subobscura*. In the analysis of *ND5* gene fragment this previous research found three sequences with six, seven and eight nucleotide change compared to S2, which is one of the two main haplotypes. All three haplotypes were in the rut of *ND5* haplotype network. These sequences could be ancient relicts that have survived to date in the Southern Europe after glaciation. The analyzed island populations were to some extent protected from gene flow allowing these variants to be preserved. Populations from our research may have survived more severe reduction in population size during last glaciation, leading to absence of these relict haplotypes. Among the haplotypes reported here, the highest number of nucleotide changes (three), compared to the most frequent sequence C1, had haplotype C5. The Balkan Peninsula with its specific habitats may have contained glacial refugia for *D. subobscura*, but this effect cannot be observed in the present sequences possibly due to high migration rates in fruit fly (AYALA *et al.,* 1989; STAMENKOVIĆ-RADAK *et al.,* 2012). The singletons found in this study are derived from the main haplotype by one nucleotide change in most of the cases and are of recent origin. Both CASTRO *et al.,* (2010) and CHRISTIE *et al.,* (2010) pointed to the seasonal bottlenecks in cold winters and dry summers which are followed by population expansion, as main reason why *D. subobscura* populations have not reached the equilibrium for mtDNA haplotypes. The pattern of the observed nucleotide variation in *Cyt b* gene that is observed is our research is in agreement with the hypothesis of seasonal contractions and expansions of the census in *D. subobscura*.

The data presented in this study indicate possible presence of the *Cyt b* variants with mildly deleterious effect on fitness in *D. subobscura*, but also point to the seasonal fluctuations in population size influencing the observed mtDNA pattern in this species.

ACKNOWLEDGEMENTS

This work was supported by grant no. 173012 of the Ministry of Education, Science and Technological Development of the Republic of Serbia.

Received, May 17th, 2018
Accepted November 18th, 2018

REFERENCES

AFONSO, J.M., A., VOLZ, M., HERNANDEZ, H., RUTTKAY, M., GONZALEZ, J.M., LARRUGA, V.M., CABRERA, D., SPERLICH (1990): Mitochondrial DNA variation and genetic structure in Old-World populations of *Drosophila subobscura*. Mol. Biol. Evol., *7*(2): 123–142.

ARNQVIST, G., Z., KURBALIJA NOVIČIĆ, J.A., CASTRO, A., SAYADI (2016): Negative frequency dependent selection on sympatric mtDNA haplotypes in *Drosophila subobscura*. Hereditas, *153*:15.

AYALA, F.J., L., SERRA, A., PREVOSTI (1989): A grand experiment in evolution: the *Drosophila subobscura* colonization of the Americas. Genome, *31*(1): 246-255.

BALLARD, J.W.O., M., KREITMAN (1994): Unraveling selection in the mitochondrial genome of *Drosophila*. Genetics, *138*(3): 757–772.

CASTRO, J.A., M., RAMON, A., PICORNELL, A., MOYA (1999): The genetic structure of *Drosophila subobscura* populations form the islands of Majorca and Minorca (Balearic Islands, Spain) based on allozymes and mitochondrial DNA. Heredity, *83*(3): 271–279.

CASTRO, J.A., P., OLIVER, J.S., CHRISTIE, A., PICORNELL, M., RAMON, A., MOYA (2003): Assortative mating and fertility in two *Drosophila subobscura* strains with different mitochondrial DNA haplotypes. Genetica, *119*(3): 295–301.

CASTRO, J.A., E., BARRIO, A., GONZÁLEZ, A., PICORNELL, M.M., RAMON, A., MOYA (2010): Nucleotide diversity of a *ND5* fragment confirms that population expansion is the most suitable explanation for the mtDNA haplotype polymorphism of *Drosophila subobscura*. Genetica, *138*(8): 819–829.

CHRISTIE, J.S., J.A., CASTRO, P., OLIVER, A., PICORNELL, M.M., RAMON, A., MOYA (2004): Fitness and life-history traits of the two major mitochondrial DNA haplotypes of *Drosophila subobscura*. Heredity, *93*(4): 371–378.

CHRISTIE, J.S., A., PICORNELL, A., MOYA, M.M., RAMON, J.A., CASTRO (2010): Dynamics of the mtDNA haplotype variability in a *Drosophila subobscura* population over a two year period. Open Evol. J., *4*: 23–30.

CHRISTIE, J.S., A., PICORNELL, A., MOYA, M.M., RAMON, J.A., CASTRO (2011): Mitochondrial DNA effects on fitness in *Drosophila subobscura*. Heredity, *107*(3): 239–245.

CLEMENT, M., Q., SNELL, P., WALKE, D., POSADA, K., CRANDALL (2002): TCS: estimating gene genealogies. Proc. 16th Int. Parallel Distrib. Process Symp., *2*:184.

EXCOFFIER, L., H.E.L., LISCHER (2010): Arlequin suite ver. 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour., *10*(3): 564-567.

EYRE-WALKER, A. (2002): Changing Effective Population Size and the McDonald-Kreitman Test. Genetics, *162*(4): 2017–2024.

FAY, J.C., C.I., WU (2000): Hitchhiking under positive Darwinian selection. Genetics, *155*(3): 1405-1413.

FU, Y-X., W.H., LI (1993): Statistical tests of neutrality of mutations. Genetics, *133*(3): 693–709.

FU, Y-X. (1997): Statistical tests of neutrality of mutations against population growth hitchhiking and background selection. Genetics, *147*(2): 915-925.

GAO, J.J., H.A., WATABE, T., AOTSUKA, J.F., PANG, Y.P., ZHANG (2007): Molecular phylogeny of the *Drosophila obscura* species group, with emphasis on the Old World species. BMC Evol. Biol., *7*:87.

HALL, T. (1999): BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Sym. Ser., *41*: 95-98.

JELIĆ, M., J.A., CASTRO, Z., KURBALIJA NOVIČIĆ, B., KENIG, D., DIMITRIJEVIĆ, M., SAVIĆ VESELINOVIĆ, M., JOVANOVIĆ, D., MILOVANOVIĆ, M., STAMENKOVIĆ-RADAK, M., ANDJELKOVIĆ (2012): Absence of linkage disequilibria between chromosomal arrangements and mtDNA haplotypes in natural populations of *Drosophila subobscura* from the Balkan Peninsula. Genome, *55*(3): 214-221.

JELIĆ, M., G., ARNQVIST, Z., KURBALIJA NOVIČIĆ, B., KENIG, M., TANASKOVIĆ, M., ANĐELKOVIĆ, M., STAMENKOVIĆ-RADAK (2015): Sex-specific effects of sympatric mitonuclear variation on fitness in *Drosophila subobscura*. BMC Evol. Biol., *15*:135.

KURBALIJA NOVIČIĆ, Z., E., IMMONEN, M., JELIĆ, M., ANĐELKOVIĆ, M., STAMENKOVIĆ-RADAK, G., ARNQVIST (2015): Within-population genetic effects of mtDNA on metabolic rate in *Drosophila subobscura*. J. Evol. Biol., *28*(2): 338–346.

LATORRE, A., C., HERNÁNDEZ, D., MARTÍNEZ, J.A., CASTRO, M.M., RAMÓN, A., MOYA (1992): Population structure and mitochondrial DNA gene flow in Old World populations of *Drosophila subobscura*. Heredity, *68*(1): 15–24.

LEIGH, J.W., D., BRYANT (2015): PopART: Full-feature software for haplotype network construction. Methods Ecol. Evol., *6*(9):1110–1116.

LIBRADO, P., J., ROZAS (2009): DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics, *25*(11): 1451-1452.

MARTINEZ, D., A., MOYA, A., LATORRE, A., FERERES (1992): Mitochondrial DNA variation in *Rhopalosiphum padi* (Homoptera: Aphididae) populations from four Spanish localities. Ann. Entomol. Soc. Am., *85*(2): 241-246.

MCDONALD, J.H., M., KREITMAN (1991): Adaptive protein evolution at the Adh locus in *Drosophila*. Nature, *351*(6328): 652-654.

MEIKLEJOHN, C.D., K.L., MONTOOTH, D.M., RAND (2007): Positive and negative selection on the mitochondrial genome. Trends Genet., *23*(6): 259–263.

NACHMAN, M.W., W.M., BROWN, M., STONEKING, C.F., AQUADRO (1996): Nonneutral mitochondrial DNA variation in humans and chimpanzees. Genetics, *142*(3): 953–963.

NACHMAN, M.W. (1998): Deleterious mutations in animal mitochondrial DNA. Genetica, *102-103*(1-6):61-69.

OLIVER, P., J.A., CASTRO, A., PICORNELL, M.M., RAMON, E., SOLÉ, J., BALANYÀ, L., SERRA, A., LATORRE, A., MOYA (2002): Linkage disequilibria between mtDNA haplotypes and chromosomal arrangements in a natural population of *Drosophila subobscura*. Heredity, *89*(2): 133–138.

RAND, D.M., M., DORFSMAN, L.M., KANN (1994): Neutral and non-neutral evolution of *Drosophila* mitochondrial DNA. Genetics, *138*(3): 741–756.

RAND, D.M., L.M., KANN (1996): Excess amino acid polymorphism in mitochondrial DNA: contrasts among genes in *Drosophila* mice and humans. Mol. Biol. Evol., *13*(6): 735–748.

RAND, D.M., L.M., KANN (1998): Mutation and selection at silent and replacement sites in the evolution of animal mitochondrial DNA. Genetica, *102-103*(1-6): 393-407.

ROGERS, A., H. HARPENDING (1992): Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol., *9*(3): 552-569.

SIMÕES, P.M., G., MIALDEA, D., REISS, M.-F., SAGOT, S., CHARLAT (2011): *Wolbachia detection*: an assessment of standard PCR protocols. Mol. Ecol. Resour., *11*(3): 567-572.

STAMENKOVIĆ-RADAK, M., M., JELIC, Z., KURBALIJA NOVIČIČ, B., KENIG, M., TANASKOVIC, M., ANDJELKOVIĆ (2012): Balkan glacial history and modern *Drosophila subobscura* population genetics. Evol. Ecol. Res., *14*: 839-858.

TAJIMA, F. (1989): Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics, *123*(3): 585-595.

TEMPLETON, A.R. (1996): Contingency tests of neutrality using intra/interspecific gene trees: the rejection of neutrality for the evolution of the mitochondrial cytochrome oxidase II gene in the hominoid primates. Genetics, *144*(3): 1263–1270.

WEINREICH, D.M., D.M. RAND (2000): Contrasting patterns of nonneutral evolution in proteins encoded in nuclear and mitochondrial genomes. Genetics, *156*(1): 385-399.

NUKLEOTIDNI DIVERZITET *Cyt b* GENA VRSTE *Drosophila subobcura*

Pavle ERIĆ[1], Mihailo JELIĆ[2], Marija SAVIĆ VESELINOVIĆ[2], Bojan KENIG[1], Marko ANĐELKOVIĆ[1,2,3], Marina STAMENKOVIĆ-RADAK[1,2]

[1] Institut za biološka istraživanja "Siniša Stanković", Univerzitet u Beogradu, Beograd
[2] Biološki fakultet, Univerzitet u Beogradu, Beograd
[3] Srpska akademija nauka i umetnosti, Beograd

Izvod

Analizirana je varijabilnost prisustva restrikcionih mesta celokupne mitohondrijske DNK i nukleotidne sekvence *Cyt b* gena kod *Drosophila subobscura* iz jugoistočne Srbije. Cilj je bio rasvetljavanje uloge evolucionih činilaca u oblikovanju varijabilnosti mitohondrijskog genoma ove vrste. Uzorci su skupljeni sa dva lokaliteta u podnožju Stare planine. Nisu dobijene značajne genetičke razlike među njima, odnosno najveći udeo variranja je bio prisutan u okviru lokaliteta. Prema prisustvu restrikcionih mesta utvrđeno je prisustvo dva česta haplotipa i nekoliko retkih. Analizom nukleotidne sekvence *Cyt b* gena uočen je veći broj haplotipova, od kojih je jedan bio visoke učestalosti. Većina retkih haplotipova se razlikuju od čestih u prisustvu samo jedne nukleotidne promene. Iako je moguće da neke od nukleotidnih razlika između haplotipova utiču na razlike u adaptivnoj vrednosti, uočena varijabilnost mitohodrijske DNK prati obrazac sezonske fluktuacije veličine populacija.

Primljeno 17.V.2018.
Odobreno 18. XI. 2018.