

INTRA-SPECIES DIFFERENTIATION AMONG *DROSOPHILA SUBOBSCURA* FROM DIFFERENT HABITATS IN SERBIA

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Abstract — Adaptation to different environmental conditions is a natural phenomenon that potentially leads to population subdivision. We surveyed genetic differentiation in inversion polymorphism within populations of *Drosophila subobscura* sampled in three ecologically different forest communities. The analysis of inversion polymorphism revealed significant differences between some pairs of samples in some gene arrangement frequencies of the A, U, and E chromosomes and some karyotype combination frequencies of the U chromosome, but significant differentiation within populations was not observed. It cannot be decided which evolutionary forces are responsible for the observed variability in inversion polymorphism.

Key words: *Drosophila subobscura*, inversion polymorphism, habitat variability

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INTRODUCTION

The Palaearctic species *Drosophila subobscura* shows rich inversion polymorphism in all five acrocentric chromosomes of the set. In contrast to allozymes, microsatellites, and mtDNA haplotypes (Latorre et al., 1992; Pascual et al., 2001), this type of polymorphism displays clear-cut geographical variation (Krimbas, 1993; Balanya et al., 2003). Temporal variation of chromosomal polymorphism was also proven to exist in natural populations of this species. These studies included diurnal (de Frutos and Prevosti, 1984; Gosteli, 1991), seasonal (de Frutos and Prevosti, 1984; Rodriguez-Trelles et al., 1996), and annual (de Frutos and Prevosti, 1984; Orengo and Prevosti, 1996; Anđelković et al., 2007) monitoring of inversion polymorphism change. All of these aspects of variability in *D. subobscura* inversion polymorphism are to a certain degree associated with the variety and dynamics of ecological factors, but since its high level of variability is quite stable it is classified as “semi rigid” or “semi flexible” (Sperlich and Feuerbach, 1966). Since inversion

polymorphism responds to climatic changes, both spatially and annually, it is reasonable to assume that environmental differences in neighboring habitats can lead to local adaptation and microhabitat variability of inversion polymorphism.

The association between gene arrangement frequencies and environmental factors in different microhabitats was shown previously (Anđelković et al., 2003). In addition to inversion polymorphism, monitoring of microhabitat variability in *D. subobscura* species revealed differences in quantitative parameters such as variability in ovariole number (Savić et al., 2008), and also in body size parameters such as thorax size, wing size, and wing loading (Stamenković-Radak et al., 2008). Those studies included comparisons between two samples taken from only two environmentally different habitats and always from the same location (Mt. Goč in Central Serbia). Since the Balkan Peninsula is characterized by long-standing and extensive ecological diversity, it provides opportunities for more detailed research of within-population differentiation of *D.*

subobscura from localities that are comprised of ecologically different microhabitats. Therefore, the aim of this research was to determine the level of local adaptation to different environmental conditions, which is constantly counteracting the gene flow due to the closeness of habitats.

MATERIALS AND METHODS

Population samples

Localized differentiation of *D. subobscura* was studied within two populations. One population lives in the southeastern part of the Deliblato Sands, the largest sandy terrain in Europe, situated between 44° 48' - 45° 12' N and 20° 51' - 21° 20' E in Northern Serbia (at an altitude of 240 m). Steppe-forest mosaic habitats and different soil composition (black, brown, and yellow sand) are characteristics of this region. Flies were collected simultaneously in mid-June 2006 using fermented fruit traps from three forest communities topographically about 3 km apart: *Pine* (with *Pinus nigra* as the dominant tree), *Elm* (with *Ulmus laevis* as the dominant tree), and *Oak* (with *Quercus robur* as the dominant tree). These three forests have distinctive microclimates. Sparse trees and direct sunlight causes *Pine* to be the warmest and most insolated habitat. *Oak* is a dense forest with lower temperature and higher humidity than *Pine*. Diurnal changes of the humidity are discreet in *Oak*. *Elm* is moderate compared to the former two localities in terms of ecological factors.

The other population of *D. subobscura* was sampled on Mt. Goč (43° 30' - 43° 35' N and 20° 31' - 20° 58' E) in Central Serbia. Flies were collected simultaneously in mid-June 2007 using fermented fruit traps from three forest communities topographically about 5 km apart: *Beech* (with *Fagus sylvatica* as the dominant tree), *Pine* (with *Pinus nigra* as the dominant tree), and *Oak* (with *Quercus petraea* as the dominant tree), at about 800 m above sea level. Since local differences in topography and soil composition, as well as distribution of dominant trees, makes microclimates considerably different (Gajić, 1984), these three forest communities represent three ecologically different habitats. *Beech* (875 m) has the highest humidity with great vegetation

coverage. *Oak* (787 m) has more sparse trees and is slightly warmer. *Pine* (690 m) has the least vegetation coverage. It is the warmest habitat.

Inversion polymorphism data analysis

Analysis of inversion polymorphism was carried out for captured wild males. The males were individually crossed with virgin females from the Kusnacht laboratory stock, which is homozygous for standard gene arrangement at all five large chromosomes. Salivary glands from third-instar larvae were squashed and chromosomes stained with aceto-orcein solution. Eight larvae were analyzed from the progeny of each of the crosses performed. For cytological analysis of gene arrangements, the chromosome map of Kunze-Muhl and Müller (1958) was used. The designation of gene arrangements follows that of Kunze-Muhl and Sperlich (1955). The analysis included a total of 100 males (800 autosomes and 100 sex chromosomes) from the Deliblato population. Thirty-five males were analyzed from *Pine* (280 autosomes and 35 sex chromosomes), 34 from *Elm* (272 autosomes and 34 sex chromosomes), and 31 from *Oak* (248 autosomes and 31 sex chromosomes).

A total of 90 males (720 autosomes and 90 sex chromosomes) were analyzed from the Goč population. The analysis included 30 males (240 autosomes and 30 sex chromosomes) from each forest community.

Z-Statistics (Zar, 1999) was used to assess the differences between frequencies of gene arrangements and karyotypes individually, between pairs of analyzed samples. The G-test (Sokal and Rohlf, 1980) was used to determine population subdivision by determining the homogeneity of gene arrangement and karyotype frequencies between pairs of samples and all samples together from different forest communities on all chromosomes and autosomes, respectively. Sequential Bonferroni correction was applied to adjust for multiple pairwise comparisons.

Wright's F-statistics (Wright, 1965, 1978) has proven to be a very useful tool in elucidating the pattern and extent of genetic variation residing within and among natural populations of different

plant (Aguirre-Planter et al., 2000; Chung et al., 2004) and animal (Aranguren-Mendez et al., 2002) species, including *D. subobscura* (Pascual et al., 2001). Population structure in our paper was analyzed using Weir and Cockerham's methods (1984), implemented in the FSTAT (Goudet, 2001) computer program (version 2.9.3.2). Significance of the F_{IS} , F_{ST} and F_{IT} indices was tested based on 1000 permutations of gene arrangements among individuals within samples, genotypes among samples, and gene arrangements among samples, respectively. While testing the significance of F indices over all autosomes using permutation, more weight was given to chromosomes with higher polymorphism. Bootstrap confidence intervals (95%) were constructed and the overall chromosome F statistics were considered significant when confidence intervals did not overlap zero. Since the bootstrapping procedure requires more than four loci (Raymond and Rousset, 1995) and since the O chromosome can be subdivided into two segments with no overlapping inversions (with the exception of the rare O_{25}), the O chromosome was observed as two separate units in calculating F statistics. These calculations were also made using the FSTAT program (Goudet, 2001).

Inversion polymorphism parameters (degree of heterozygosity, inversion density and, index of free recombination) were derived from arrangement frequencies according to the description of Krimbas (1993).

RESULTS

The obtained frequencies of gene arrangements on five acrocentric chromosomes, degree of heterozygosity, inversion density, and index of free recombination are presented in Table 1. The obtained karyotype frequencies are presented in Table 2.

Inversion polymorphism parameters (degree of heterozygosity, inversion density, and index of free recombination) were similar between all six samples. The indices of free recombination are higher than values characteristic of the Balkan region (Krimbas, 1993).

Differences in the frequencies of individual gene

arrangements and karyotypes between samples from different forest communities within populations are presented in Tables 3 and 4.

Comparisons of gene arrangement frequencies between samples from the Deliblato Sands gave significant differences for the E_8 ($z = -3.70$; $p < 0.01$) gene arrangement between *Elm* and *Oak*. No significant differences were found between the other two pairwise comparisons. Significant differences in karyotype frequencies were found for U_{1+2}/U_{1+2+6} ($z = -3.33$; $p < 0.05$) between *Pine* and *Elm*, U_{1+2}/U_{1+2+6} ($z = -3.93$; $p < 0.001$), and U_{1+2+6}/U_{1+2+6} ($z = 3.60$; $p < 0.05$) between *Pine* and *Oak*.

In the Goč population, significant differences of gene arrangement frequencies were found between *Beech* and *Oak* for U_{1+2} ($z = 3.63$; $p < 0.01$), and *Est* ($z = 3.24$; $p < 0.05$); and between *Pine* and *Oak* for *Ast* ($z = -3.66$; $p < 0.01$), A_1 ($z = 3.38$; $p < 0.05$), and U_{1+2} ($z = 3.37$; $p < 0.05$). No significant differences were found in karyotype frequency comparisons for the Goč population.

The G-test did not reveal significant differences in the distribution of gene arrangements and karyotypes between samples from three forest communities for either single or all chromosomes in either population. Results of the G-test were not significant for either pairwise comparisons or comparisons of all three samples within populations.

The results of Wright's F-statistics are presented in Table 5. In the Deliblato population, the coefficients of inbreeding within three analyzed samples (F_{IS}) were generally positive and significant for U ($F_{IS} = 0.188$; $p < 0.01$), E ($F_{IS} = 0.282$; $p < 0.001$) chromosomes, the OII chromosome segment ($F_{IS} = 0.152$; $p < 0.05$), and overall autosomes ($F_{IS} = 0.166$; $p < 0.001$). The total inbreeding coefficient (F_{IT}) estimates were also generally positive and significant for U ($F_{IT} = 0.189$; $p < 0.01$), E ($F_{IT} = 0.291$; $p < 0.001$) chromosomes, and overall autosomes ($F_{IT} = 0.168$; $p < 0.001$). In the Goč population, all F_{IT} values were positive and significant for the OII chromosome segment ($F_{IT} = 0.091$; $p < 0.05$). The Goč population shows significant F_{IS} only for the OII chromosome segment ($F_{IS} = 0.088$; $p < 0.05$). Values of F_{ST} did

Table 1. Gene arrangement frequency (%) and inversion polymorphism parameters in six *D. subobscura* samples from different forest communities; degree of heterozygosity (HZ); index of free recombination (IFR); inversion density (ID); number of males in the analysis (n).

Gene arrangement (%)	Deliblato Sands				Goč	
	<i>Pine</i> (n=35)	<i>Elm</i> (n=34)	<i>Oak</i> (n=31)	<i>Beech</i> (n=30)	<i>Pine</i> (n=30)	<i>Oak</i> (n=30)
A _{st}	31.43	26.47	32.26	43.33	36.67	70.00
A ₁	57.14	58.82	54.84	50.00	53.33	23.33
A ₂	11.43	14.71	12.90	6.67	10.00	6.67
J _{st}	14.29	25.00	25.81	18.33	21.67	20.00
J ₁	85.71	75.00	74.19	81.67	78.33	80.00
U _{st}	22.86	26.47	20.97	3.33	6.67	11.67
U ₁₊₂	41.43	36.76	54.84	66.67	65.00	43.33
U ₁₊₂₊₆	35.71	36.76	24.19	30.00	28.33	45.00
E _{st}	28.57	42.65	27.42	35.00	21.67	16.67
E ₈	32.86	22.06	43.55	16.67	33.33	31.67
E ₁₊₂₊₉	38.57	35.29	27.42	48.33	45.00	51.67
E ₁₊₂₊₉₊₁₂	0.00	0.00	1.61	0.00	0.00	0.00
O _{st}	21.43	14.71	24.20	10.00	13.33	15.00
O ₆	0.00	0.00	0.00	0.00	0.00	1.67
O ₃₊₄	47.14	50.00	43.55	43.33	51.67	50.00
O ₃₊₄₊₁	27.14	33.82	25.81	40.00	35.00	26.67
O ₃₊₄₊₂	4.29	1.47	6.45	6.67	0.00	6.67
IFR (%)	84.16	83.08	82.41	85.13	83.78	83.13
ID	5.94	5.56	5.70	6.66	6.73	6.73
HZ	0.44	0.49	0.54	0.47	0.52	0.52

not show significant deviation from zero for either particular chromosomes or overall chromosomes within either population. Bootstrapping gave results that are in agreement with permutation tests except for F_{IS} and F_{IT} values for overall autosomes within the Goč population, where these indices did not overlap zero.

DISCUSSION

In general, the pattern of inversion polymorphism of *D. subobscura* populations from the Deliblato Sands and Mt. Goč is consistent with hitherto observed inversion polymorphism for the area of the south-eastern margin of the Central European range of the species (Krimbas, 1993), except for the O_{3+4+2}

arrangement, which was found in relatively high frequencies in five analyzed samples. This arrangement, which is characteristic of populations from Southern Europe (Krimbas, 1993), was registered for the first time in the Goč population in 2003 in frequencies of less than 4% (Anđelković et al., 2007; Rašić et al., 2008). The given arrangement is also registered in the Deliblato Sands, which are at a higher latitude than Mt. Goč. These data support the “southern effect” hypothesis, which suggests that chromosomal inversion polymorphism of *D. subobscura* responds to climatic change (Rodriguez-Trelles et al., 1996; Balanya et al., 2006; Stamenković-Radak et al., 2008).

Many observations published so far strongly

Table 2. Karyotype frequency (%) in six *D. subobscura* samples from different forest communities; number of males in the analysis (n).

Karyotype (%)	Deliblato Sands				Goč	
	Pine (n=35)	Elm (n=34)	Oak (n=31)	Beech (n=30)	Pine (n=30)	Oak (n=30)
J_{st}/J_{st}	0.00	5.88	3.23	3.33	6.67	3.33
J_{st}/J_1	28.57	38.24	45.16	30.00	30.00	33.33
J_1/J_1	71.43	55.88	51.61	66.67	63.33	63.33
U_{st}/U_{st}	5.71	8.82	9.68	0.00	0.00	3.33
U_{st}/U_{1+2}	20.00	11.76	16.13	3.33	10.00	3.33
U_{st}/U_{1+2+6}	14.29	23.53	9.68	3.33	3.33	13.33
U_{1+2}/U_{1+2}	28.57	17.65	29.03	46.67	40.00	20.00
U_{1+2}/U_{1+2+6}	5.71	26.47	32.26	36.67	40.00	43.33
U_{1+2+6}/U_{1+2+6}	25.71	11.76	3.23	10.00	6.67	16.67
E_{st}/E_{st}	17.14	26.47	12.90	13.33	3.33	3.33
E_{st}/E_8	5.71	11.76	19.35	10.00	23.33	6.67
E_{st}/E_{1+2+9}	17.14	20.59	9.68	33.33	13.33	20.00
E_8/E_8	17.14	11.76	22.58	0.00	6.67	13.33
E_8/E_{1+2+9}	25.71	8.82	22.58	23.33	30.00	30.00
E_{1+2+9}/E_{1+2+9}	17.14	20.59	9.68	20.00	23.33	26.67
$E_{1+2+9}/E_{1+2+9+12}$	0.00	0.00	3.23	0.00	0.00	0.00
O_{st}/O_{st}	8.57	2.94	9.68	3.33	3.33	6.67
O_{st}/O_{3+4}	22.86	11.76	25.81	3.33	13.33	3.33
O_{st}/O_{3+4+1}	2.86	11.76	3.23	3.33	6.67	10.00
O_{st}/O_{3+4+2}	0.00	0.00	0.00	6.67	0.00	3.33
O_6/O_{3+4+2}	0.00	0.00	0.00	0.00	0.00	3.33
O_{3+4}/O_{3+4}	20.00	29.41	9.68	26.67	26.67	30.00
O_{3+4}/O_{3+4+1}	25.71	29.41	32.26	26.67	36.67	30.00
O_{3+4}/O_{3+4+2}	5.71	0.00	6.45	3.33	0.00	6.67
O_{3+4+1}/O_{3+4+1}	11.43	11.76	9.68	23.33	13.33	6.67
O_{3+4+1}/O_{3+4+2}	2.86	2.94	0.00	3.33	0.00	0.00
O_{3+4+2}/O_{3+4+2}	0.00	0.00	3.23	0.00	0.00	0.00

suggest that different inversion arrangements carry various alleles that are differently favored in diverse environmental conditions and prove in most cases to be the major factors determining the frequencies of inversion arrangements in natural populations of *D. subobscura* (Anđelković et al., 2003). Temporal and spatial variation of inversion polymorphism has also been studied in natural populations of

Drosophila buzzatii and revealed coexistence of different karyotypes in different environmental conditions (Fernandez Iriarte et al., 1999). There is also evidence of micro-geographic variation of inversion polymorphism in natural populations of *Drosophila pseudoobscura* (Salceda and Espinoza-Velazquez, 2006). The results presented here further emphasize the relationship between gene arrangement

Table 3. Differences in the frequencies of individual gene arrangements of *D. subobscura* between samples from different forest communities before (in parentheses) and after Bonferroni multiple test correction; Z-test values are given only for significant comparisons; all comparisons that were significant after correction had $p < 0.001$ before correction.

Gene arrangement	Deliblato Sands			Goč		
	<i>Pine/Elm</i>	<i>Pine/Oak</i>	<i>Elm/Oak</i>	<i>Beech/Pine</i>	<i>Beech/Oak</i>	<i>Pine/Oak</i>
A _{st}					(- 2.95**)	- 3.66**
A ₁					(3.03**)	3.38*
A ₂						
J _{st}	(- 2.24*)	(- 2.35*)				
J ₁	(2.24*)	(2.35*)				
U _{st}					(- 2.45*)	
U ₁₊₂		(- 2.18*)	(- 2.93**)		3.63**	3.37*
U ₁₊₂₊₆		(2.04*)	(2.20*)		(- 2.40*)	(- 2.68**)
E _{st}	(- 2.44*)		(2.57*)	(2.29*)	3.24*	
E ₈	(2.01*)		- 3.70**	(- 2.98**)	(- 2.71**)	
E ₁₊₂₊₉						
E ₁₊₂₊₉₊₁₂						
O _{st}						
O ₆						
O ₃₊₄						
O ₃₊₄₊₁					(2.19*)	
O ₃₊₄₊₂			(- 2.09*)	(2.88**)		(- 2.88**)

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

frequencies and local adaptation to ecologically different microhabitats in *Drosophila* species. However, genetic drift can also generate gene arrangement frequency differences between local populations of restricted size. Study of populations from the Goč locality over a number of years (Stamenković-Radak et al., 2008) showed a significant decrease in population size in some years. But, since inversion polymorphism responds to climatic variation, both annually (de Frutos and Prevosti, 1984; Orengo and Prevosti, 1996; Anđelković et al., 2007) and spatially (Krimbas, 1993), it is likely that microclimatic conditions in a given ecological niche differentially select some gene arrangements due to their adaptive significance. A certain degree of association of this kind of polymorphism with environmental variability corresponds to the flexible in contrast

to the rigid (Dobzhansky, 1962) type of polymorphism. Regardless of which factors contribute to the observed differences, closeness of habitats disrupts localized differentiation due to the high dispersion capacity of this species (Serra et al., 1987). The obtained G-test comparisons and F-statistics were in agreement. Values of F_{ST} did not show differentiation within populations. The observed F_{IS} and F_{IT} values, which were significant for particular chromosomes, may indicate a role for non-random mating in reducing the observed heterozygosity.

Although the observed differences in gene arrangement frequencies are slight, they are not negligible, since more genetic differences, which are adaptive, could exist within inversions. Rašić et al. (2008) showed that differences in gene arrangement frequencies between *D. subobscura* collected from

Table 4. Differences in the frequencies of individual karyotypes of *D. subobscura* between samples from different forest communities before (in parentheses) and after Bonferroni multiple test correction; Z-test values are given only for significant comparisons; all comparisons that were significant after correction had $p < 0.001$ before correction.

Karyotype	Deliblato Sands			Goč		
	<i>Pine/Elm</i>	<i>Pine/Oak</i>	<i>Elm/Oak</i>	<i>Beech/Pine</i>	<i>Beech/Oak</i>	<i>Pine/Oak</i>
J_{st}/J_{st}	(- 2.06*)					
J_{st}/J_1		(- 1.98*)				
J_1/J_1		(2.34*)				
U_{st}/U_{st}						
U_{st}/U_{1+2}						
U_{st}/U_{1+2+6}			(2.11*)		(- 1.98*)	(- 1.98*)
U_{1+2}/U_{1+2}					(2.70**)	(2.03*)
U_{1+2}/U_{1+2+6}	- 3.33*	- 3.93***				
U_{1+2+6}/U_{1+2+6}	(2.09*)	3.60*				
E_{st}/E_{st}				(1.98*)	(1.98*)	
E_{st}/E_8		(- 2.40*)				
E_{st}/E_{1+2+9}				(2.59**)		
E_8/E_8				(- 2.13*)	(- 3.06**)	
E_8/E_{1+2+9}	(2.61**)		(- 2.17*)			
E_{1+2+9}/E_{1+2+9}						
$E_{1+2+9}/E_{1+2+9+12}$						
O_{st}/O_{st}						
O_{st}/O_{3+4}			(- 2.06*)	(- 1.98*)		(1.98*)
O_{st}/O_{3+4+1}	(- 2.02*)					
O_{st}/O_{3+4+2}				(2.03*)		
O_6/O_{3+4+2}						
O_{3+4}/O_{3+4}						
O_{3+4}/O_{3+4+1}						
O_{3+4}/O_{3+4+2}	(2.00*)		(- 2.13*)			(- 2.03*)
O_{3+4+1}/O_{3+4+1}					(2.56*)	
O_{3+4+1}/O_{3+4+2}						
O_{3+4+2}/O_{3+4+2}						

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

two different habitats were higher after several generations of inbreeding in laboratory conditions than had been registered before inbreeding. The genetic systems differed in structure and integrity of the genome in the sense that the effect of homozygosity appeared to be habitat- and chromosome-specific.

The results of this paper show that three forest communities in both analyzed populations cannot be considered as subpopulations of *D. subobscura*, although the inversion polymorphism in *D. subobscura*, with its microhabitat variability, could be a potential genetic marker of population fragmenta-

Table 5. F-Statistics (Wright, 1965) following the method of Weir and Cockerham (1984) for four polymorphic autosomes within the studied populations.

Chromosome	Deliblato Sands			Goč		
	F _{IT}	F _{ST}	F _{IS}	F _{IT}	F _{ST}	F _{IS}
J	- 0.087	0.011	- 0.100	0.066	- 0.018	0.082
U	0.189**	0.001	0.188**	0.042	0.034	0.008
E	0.291***	0.013	0.282***	0.008	0.013	- 0.004
OI	0.174*	- 0.008	0.181	0.233	- 0.011	0.242
OII	0.142	- 0.011	0.152*	0.091*	0.003	0.088*
over all autosomes	0.168***	0.002	0.166***	0.067	0.008	0.059
Bootstrapping over chromosomes	0.046 0.248	- 0.007 0.010	0.041 0.243	0.029 0.132	- 0.010 0.023	0.012 0.129

Significance of deviation from zero (*p < 0.05, **p < 0.01, ***p < 0.001); bootstrapping confidence interval 95%.

tion due to environmental change. The observed localized differentiation needs to be further analyzed with different genetic markers.

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ГЕНЕТИЧКА ДИФЕРЕНЦИЈАЦИЈА УНУТАР ВРСТЕ *DROSOPHILA SUBOBSCURA* СА РАЗЛИЧИТИХ СТАНИШТА У СРБИЈИ

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Адаптација на различите срединске услове може утицати на диференцијацију у оквиру популација. У овом раду анализирана је генетичка диференцијација по инверзионом полиморфизму у оквиру популација *D. subobscura* сакупљених у три еколошки различита станишта. Утврђене су значајне разлике у учесталости појединих

хромозомских аранжмана А, У и Е хромозома, и учесталости појединих комбинација кариотинова У хромозома, али није утврђена диференцијација у оквиру популација. Разматрани су еволутивни фактори који би могли да буду одговорни за уочену варијабилност у инверзионом полиморфизму.