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GENETIC DIFFERENTIATION AND LINKAGE DISEQUILIBRIUM IN A SPATIALLY FRAGMENTED POPULATION OF *CHEILOSIA VERNALIS* (DIPTERA: SYRPHIDAE) FROM THE BALKAN PENINSULA

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Multi-locus allozyme associations and genetic differentiation in a spatially fragmented population of *Cheilosia vernalis* were analyzed based on the allelic frequencies at nine polymorphic allozyme loci. Standardized variance of allelic frequencies indicated that the spatially separate subpopulations from three different biogeographical regions on the Balkan Peninsula were a part of a structured metapopulation, with a moderately to greatly differentiated gene pool. Pairwise genetic differentiation was correlated with genetic distance between pairs of subpopulations, and both increased with increasing geographic distance. The percent of polymorphic loci pairs with significant non-random associations among alleles ranged from 33% to 50% within subpopulations. The proportion of loci pairs with non-random allelic associations was higher on the level of the total pooled population (64% for total pooled). The subdivision among the geographically separated subpopulations was analyzed by applying Ohta's method of partitioning the total variance of linkage disequilibrium. Variance components indicated that subdivided population structure coupled with limited migration, which reduced the chance of generating recombinant gametes, might be responsible for observed linkage disequilibrium.

Key words: allozymes, *Cheilosia vernalis*, genetic differentiation, linkage equilibrium, Syrphidae

INTRODUCTION

Most species are fragmented to a certain degree, depending on the environment (habitat fragmentation), species biology (dispersion, territorial behavior, character displacement), and historical events (genetic drift, founder effect, bottlenecks). Population substructuring often occurs in spatially or temporally fragmented populations. Studies of genetic diversity of spatially and/or temporally fragmented populations of species in the family Syrphidae can lead to a better understanding of microevolution processes, and shed light on forces that interacted throughout the history of a species.

Widely distributed Palaearctic species *Cheilosia vernalis*, a member of the *melanura* group of the genus *Cheilosia* (VUJIĆ 1996), has a complex population structure. The species is fragmented both spatially and temporally. Thus far, the analyses of nuclear loci and mitochondrial (mtDNA) sequences revealed a great spatial variation based on differences in genotype and allelic frequencies, and presence of rare and private alleles at several allozyme loci (MILANKOV *et al.* 2002, STÅHLS *et al.* unpubl.). Having been described 8 times under different names (PECK 1988, VUJIĆ 1996), the taxonomic status of *C. vernalis* remains unresolved. Based on the variability of the morphological traits: face in profile (facial tubercula and mouth edge), the shape, size and colour of antennae, distribution and colour of body hairs and cuticular punctuation, SPEIGHT and LUCAS (1992) and VUJIĆ (1996) suggested that *C. vernalis* included several closely related species. Since male terminalia, a conservative character in identification of many hoverflies, of different morphological variants of *C. vernalis* appeared identical no satisfactory character for subdividing the species has yet been found (SPEIGHT & LUCAS 1992).

Specimens of *C. vernalis* have been recorded in most European countries, on Caucasus Mountain, in Siberia and the Oriental region. In the northern part of its range preferred environments are dry meadows with short vegetation, old pasture, dune systems and grassy clearings in woodland, while it becomes increasingly montane in the south (STUBS & FALK 1983, TORP 1984, SPEIGHT & LUCAS 1992, SPEIGHT 2003). It has been registered at lower altitudes in the north of the Balkan Peninsula and in the Mediterranean zone (MARCOS-GARCIA 1990, VUJIĆ 1996). *C. vernalis* can be found in urban biotopes as well, such as parks, gardens and ruderal environments (BARKEMEYER 1994). The adults of *C. vernalis* have been registered from late winter (end of February) to late summer (end of August) on the Balkan Peninsula. Summer generation is occasionally registered in populations at lower altitudes, where the season is longer. During the period of activity, which is only a few days long, the adults of *C. vernalis* are often found on flowers of different species, particularly *Ficaria verna* (VUJIĆ 1996).

This paper is based on the same material used in the study of geographic variation of the spatially fragmented *C. vernalis* population from the Balkan Peninsula (MILANKOV *et al.* 2002), with the goal to elucidate genetic differentiation and multilocus allozyme associations. In order to determine the degree of population subdivision and examine evolutionary mechanisms that influenced the distribution of genetic variability and genetic divergence among the geographically separated subpopulations, OHTA's (1982) method of partitioning the total variance of linkage disequilibrium and WRIGHT's (1951) standardized variance of allelic frequencies were applied.

MATERIAL AND METHODS

Detailed descriptions of sampling locations and allozyme analysis are given in MILANKOV *et al.* (2002). Briefly, four subpopulations from different biogeographical regions of the Balkan Peninsula were collected during the period from 1995 to 1998 (Fig. 1, Table 1), and twelve izozyme loci were analyzed using 5% polyacrylamide gel electrophoresis (MUNSTERMANN 1979 modified by MILANKOV 2001). Due to the small number of collected specimens and the presence of diagnostic genotypes at the *Gpi* and *Mdh-2* loci that indicated the existence of a possibly cryptic taxon, subpopulation from Kopaonik Mountain analyzed in the study of the geographic variation (MILANKOV *et al.* 2002) and linkage equilibrium (MILANKOV *et al.* 2005) was omitted from this study.

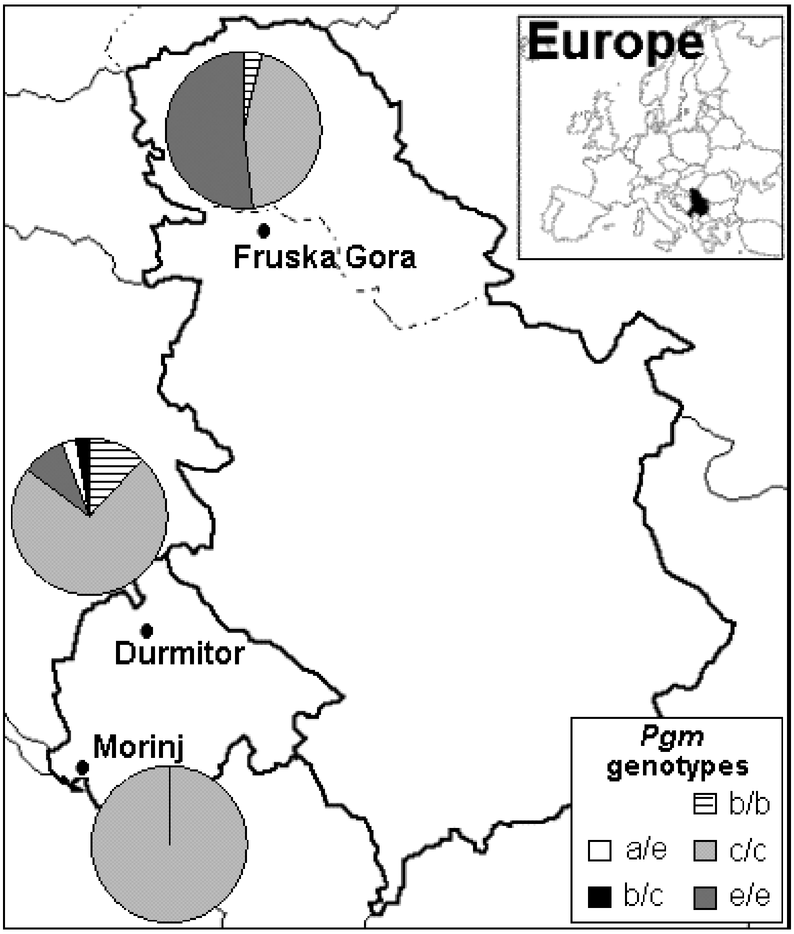

Fig. 1. Map of Serbia and Montenegro showing sampling sites for the studied subpopulations of *Chelosia vernalis*, and genotype distribution at the *Pgm* locus. The *Pgm* locus was the most variable locus in the surveyed subpopulations, and along with differences of allele frequency variances at the *Had* and *Mdh-2* loci, indicated population subdivision (MILANKOV *et al.* 2002)

Table 1. Site description, number of collected specimens, longitude (LON) and latitude (LAT) coordinates for the sampling locations within different biogeographical regions for the subpopulations of *Cheilosia vernalis* from the Balkan Peninsula

Biome:	Sampling site and description	# collected	LON	LAT
Evergreen Mediterranean maritime woodlands & maquis:				
	Morinj, Montenegro (a bay in the Adriatic sea)	46	18°40'E	43°29'N
European mostly coniferous boreal type woodlands:				
	Durmitor, Montenegro (a high Dinaric mountain)	34	19°00'E	43°11'N
South European mostly deciduous woodlands:				
	Fruška Gora, Serbia (hilly area in the Pannonian plain)	26	19°50'E	45°10'N

Allelic frequencies at polymorphic loci (0.95 criterion) were used for analyses of subpopulation genetic differentiation and linkage disequilibrium in populations of *C. vernalis.* Statistical analyses of genetic differentiation and linkage disequilibrium coefficients for multiple alleles at polymorphic loci were performed using the computer program BIOSYS-2 (SWOFFORD & SELANDER 1989). The significance of allelic frequency differences between subpopulation pairs and linkage disequilibrium in subpopulations was evaluated by the chi-square test with alpha values of 0.05 and 0.01, respectively, unless noted otherwise.

Linkage disequilibrium in *C. vernalis* subpopulations was analyzed using allelic associations of polymorphic loci. Multilocus associations were estimated for each subpopulation by pooling specimens collected in different years. Specimens from all subpopulations and across all years were pooled together for estimating linkage disequilibrium in the total population of *C. vernalis*. The distribution of genetic differentiation was analyzed using WRIGHT's (1965) F_{ST} statistic as modified by NEI (1977). All F_{ST} values were calculated using means and variances of allele frequencies weighted by sample sizes. Gene flow (Nm) was estimated from the F_{ST} values, using the equation $Nm = (1 - F_{ST})/4F_{ST}$ (WRIGHT 1978). The relationships between genetic differentiation, measured by allelic variance at particular loci (F_{ST}), and geographic (Euclidean distance between localities in kilometers) and NEI's genetic distance (D) (1978) were also examined.

Finally, the total variance of linkage disequilibrium (D^2_{IT}) was partitioned into within (D^2_{IT} and D'^2_{IT}) and between population (D^2_{IT} and D'^2_{IT}) components (OHTA, 1982) to test which of the factors was the main cause of observed deviation from random association between alleles at polymorphic enzymatic loci. For systematic associations, there is a relatively large within-population component and a relatively small between-population component, because disequilibrium is in the same direction in each population. In contrast, a large between-population component of disequilibrium is most readily attributable to nonselective effects of population subdivision or founder effects (OHTA 1982).

RESULTS

Genetic diversity

Out of 12 allozyme loci, three (*Fum, Gpd-2, Idh-1*) were monomorphic in all subpopulations, while 9 loci (*Gpi, Had, Hk-2, Hk-3, Idh-2, Mdh-1, Mdh-2, Pgm, Sod-1*) were polymorphic in at least one subpopulation (see MILANKOV *et al.*

2002). As indicated by Wright's F_{ST} parameter (Fig. 2), 10.3%, 19.5% and 30.8% of the total variance in allelic frequencies in *C. vernalis* was due to genetic differences between subpopulation pairs Durmitor-Morinj, Fruška Gora-Durmitor, and Fruška Gora-Morinj, respectively. Genetic differentiation between the Morinj and Fruška Gora subpopulations was mainly caused by the differences in the allelic frequencies at the *Pgm* ($F_{\mathrm{ST}} = 0.585$), *Had* ($F_{\mathrm{ST}} = 0.454$), *Mdh-2* ($F_{\mathrm{ST}} = 0.198$) and HK ($F_{\mathrm{ST}} = 0.174$) loci. Genetic diversity between Durmitor and Fruška Gora subpopulations was mainly the result of differences in the allelic frequencies at the *Had* ($F_{\mathrm{ST}} = 0.401$), *Pgm* ($F_{\mathrm{ST}} = 0.205$), *Mdh-2* ($F_{\mathrm{ST}} = 0.165$), and *Mdh-1* ($F_{\mathrm{ST}} = 0.158$) loci. Finally, the lowest degree of genetic changes quantified between Morinj and Durmitor was caused by the genetic changes at the *Mdh-1* ($F_{\mathrm{ST}} = 0.214$), *Pgm* ($F_{\mathrm{ST}} = 0.181$) and HK ($F_{\mathrm{ST}} = 0.178$) loci.

Pairwise F_{ST}, and genetic distances (NEI 1978) between the studied subpopulations corresponded to geographic distances, being the highest and the lowest for the most and the least geographically distant pair, respectively (Fig. 2). Analogously, the rate of migrants ranged from approximately one per two generations (*Nm* = 0.562) in Fruška Gora-Morinj subpopulation pair, to more than one (*Nm* = 1.032) and two (*Nm* = 2.177) individuals per generation in Fruška Gora-Durmitor and Durmitor-Morinj subpopulation pair, respectively.

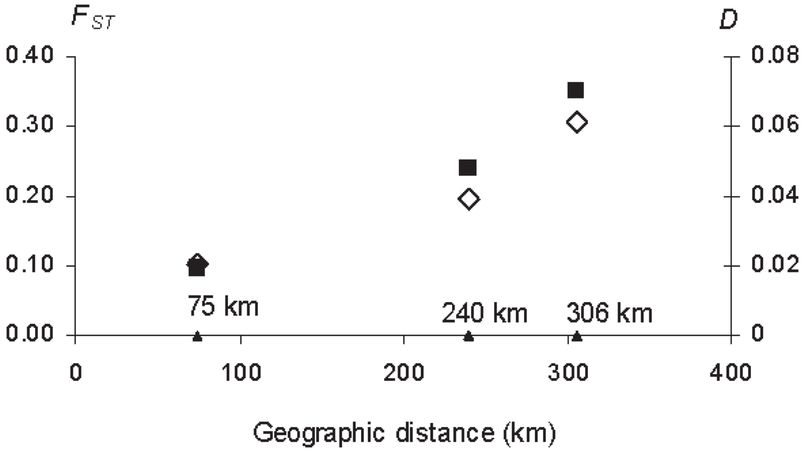

Fig. 2. Standardized variance of allelic frequencies F_{ST} (open symbols) and genetic distance D (NEI 1978) (filled symbols) plotted against corresponding geographic distance between subpopulation pairs of *Cheilosia vernalis*: Durmitor-Morinj (75 km), Fruška Gora- Durmitor (240 km), and Fruška Gora-Morinj (306 km). Pearson correlation coefficients between geographic distance and F_{ST} and D were 0.956 (p=0.190) and 0.987 (p=0.104), respectively

Linkage disequilibrium

Analysis of the polymorphic loci in populations of *C. vernalis* revealed significant associations between alleles for most loci pairs, ranging from 33% in Fruska Gora subpopulation (5 out of 15 polymorphic loci pairs were with non-random associations) to 50% in subpopulations from Durmitor (5 out of 10 polymorphic loci pairs) and Morinj (3 out of 6 polymorphic loci pairs). The proportion of loci pairs with non-random allelic associations was higher in the total pooled population than in the analysed subpopulations (Table 2). Of the 33 pairwise locus comparisons, 31 exhibited ratios of variance components characteristic for non-systematic disequilibrium ($D^2_{IS} > D^2_{ST}$ and $D'^2_{IS} > D'^2_{ST}$) in the total pooled population. Less than 15% of locus-pairs exhibited pattern typical when disequilibrium is systematic (Table 2).

The total variance of disequilibrium was calculated for each pair of loci. The average total variance in the pooled population was 0.305 (Table 3). In order to assess the contribution of geographic fragmentation to the total disequilibrium for each pair of loci, the total variance was partitioned into within- and between-population components (Table 3). The average D^2_{IS} was lower than the average D^2_{ST}, suggesting that a larger part of the total variance of disequilibrium results from deviations among subpopulations (localities) than from variation in allele frequencies within subpopulations (localities). Variance of disequilibrium of the total popula-

Table 2. Relationships and average values of disequilibrium coefficients for 33 pairs of loci in the total pooled population of *Cheilosia vernalis*

	Loci pairs	D^2_{IS}	D'^2_{IS}	D^2_{ST}	D'^2_{ST}	D^2_{IT}
i. $D^2_{IS} < D^2_{ST}$ $D'^2_{IS} < D'^2_{ST}$	31	0.010	0.311	0.136	0.007	0.318
ii. $D^2_{IS} > D^2_{ST}$ $D'^2_{IS} > D'^2_{ST}$	2	0.094	0.055	0.020	0.049	0.104

* Gpi-Sod locus-pair deviated from the above groups ($D^2_{IS} < D^2_{ST}$ $D'^2_{IS} < D'^2_{ST}$)

Table 3. Comparison of variance components of linkage disequilibrium for the total pooled population

Components		All populations
Total	D^2_{IT}	0.305±0.042
Within	D^2_{IS}	0.015±0.005
	D'^2_{IS}	0.296±0.041
Between	D^2_{ST}	0.129±0.017
	D'^2_{ST}	0.009±0.003

Entries are average variances ± SE for all 33 possible polymorphic loci pairs tion ($D_{IS}^{\prime 2}$) accounted for 95% or more of the total variance of disequilibrium (D_{IT}^{2}) in the total pooled population.

Although limited, the present study allowed for partial analysis of connection between frequency of crossing-over and sex. Sex ratios in analyzed subpopulations of *C. vernalis* were highly male biased (one female per 2.5–7.5 males). While homozygous genotypes $Had^{p/p}$, $Mdh\text{-}1^{b/b}$, $Mdh\text{-}2^{a/a}$ and $Pgm^{b/b}$ (MILANKOV *et al.* 2002) were unique to males, particular heterozygous genotypes were detected only in males from Durmitor (heterozygosity at *Gpi, Had, Idh-2* and *Pgm* loci) and Fruška Gora (*Sod*, *Gpi, Mdh-2* and *Pgm*). Furthermore, non-random associations were observed between particular genotypes in males ($Idh\text{-}2^{e/g}$ – $Gpi^{g/g}$, $Idh\text{-}2^{e/g}$ – $Pgm^{b/c}$ in Durmitor; $Gpi^{f/f}$ – $Pgm^{b/b}$ in Fruška Gora subpopulation).

DISCUSSION

In general, genetic variation among populations could be a result of both differences among ecological zones and geographic distance. Selection and genetic drift may generate interpopulation variation among geographically distant or isolated populations with minimal or no genetic exchange. Genetic divergence quantified by Wright's F_{ST} parameter indicated that the analyzed subpopulations of *C. vernalis* were a part of a structured metapopulation, with a moderately to greatly differentiated gene pool. Marginally significant correlations between F_{ST} value and genetic and geographic distances suggested independent evolution of subpopulations of *C. vernalis* on the Balkan Peninsula, probably since the Pleistocene. Based on the average genetic distance (*D*; NEI 1972), or number of allelic substitutions, it could be hypothesized that independent evolution of the subpopulations from Durmitor and Morinj lasted ca. 95 000 years (t = 5 × 106 *D*; NEI 1975). Approximate time of divergence between the Fruška Gora and other subpopulations was estimated at 240 000 years (Durmitor) and 350 000 years (Morinj) ago. Although values of *Nm* larger than 0.5 indicated that gene flow among subpopulations of *C. vernalis* could be sufficient to prevent genetic drift from causing large local genetic differentiation (SINGH & LONG 1992), given the short period of activity and the territorial behavior of adults, it is necessary to consider factors other than gene flow. For example, life history and biology of the species might also be important in generating genetic divergence and substructuring.

This study offered valuable insights into biogeographical history of the species *C. vernalis*. While spatial distribution of genotypes and gene complexes (MILANKOV *et al.* 2002) suggested natural selection as an important agent of change in the analyzed population of *C. vernali*s, unique alleles indicated possible population bottlenecks in the past. However, the majority of Ohta's variance components of linkage disequlibrium (OHTA 1982) in *C. vernali*s followed a non-systematic disequilibrium pattern, indicating that the main source of the disequilibrium in each subpopulation was population substructuring and limited migration, rather than epistatic natural selection, also supported by a high value of the local inbreeding (F_{IS}; WRIGHT 1951). In addition, a higher proportion of loci pairs with non-random allelic associations of the total pooled population compared to within subpopulations pointed to the subdivision of the total population. This pattern of linkage disequilibrium could also indicate the admixture of genetically distinct populations.

Finally, lower estimated gene flow (*Nm*) and higher values of genetic divergence suggest that the geographically distant and partially isolated subpopulations of *C. vernalis* on the Balkan Peninsula might undergo evolutionary changes more readily than continuous populations. This has important implications for management and requires strategies to maintain the genetic diversity of these subpopulations. Since recognizing and protecting genetic diversity is commonly based upon the identification of discrete populations, management units, and evolutionary significant units (MORITZ 1994, CRANDALL *et al.* 2000), estimation of genetic differences among populations of *C. vernalis* should be addressed in a potential conservation management plan for this taxon.

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