Bulletin of Insectology **67** *(2): 237-246, 2014*
ISSN 1721-8861

Microhabitat and spatial variation at HK isozyme loci in *Culex pipiens*: testing isolation by distance and isolation by ecology model

Bosiljka KRTINIĆ[1], Ljubinka FRANCUSKI[2], Vesna MILANKOV[2]
[1]*Ciklonizacija, Novi Sad, Serbia*
[2]*Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Serbia*

Abstract

Culex pipiens L. (Diptera Culicidae) is of medical and veterinary importance as vector of various bacterial, filarial and arboviral disease pathogens, and is a major pest in urban areas. Understanding the population structure and gene flow among populations is critical for public health issues such as local dispersion pattern and spread of insecticide resistance. To determine the dispersion pattern of the *Cx. pipiens* samples across the southern part of the Pannonian Plain of Central Europe (testing Isolation by distance model), and at fine-scale level, between ecologically different larval habitats (testing Isolation by ecology model), we studied hexokinase (HK) genes. Because the polymorphism of HK genes is independent of insecticide use, spatial variation at HK loci provided valuable information on population connectivity of *Cx. pipiens*. Implementing STRUCTURE statistical analysis, a lack of structuring at individual level of "urban" and "rural" samples was observed. Landscape study rejected both ecological and isolation models which implies that different selection regimes of larval habitats and geographic distance do not influence on genotype portioning at HK system. Observed high genetic connectivity among samples throughout urban and rural environments indicated that gene flow is an important factor underlying genetic structure. This study pointed out that control measures restricted to urban areas are unlikely to be effective due to high gene flow between treated and untreated populations. Hence, designing a sustainable vector control strategy for the species with detectable gene flow, such as *Cx. pipiens,* must remain a regional effort.

Key words: dispersal potential, landscape genetics, mosquito, vector control programme.

Introduction

Major public threats have been generated by increased mosquito breeding areas which influence on the increase in diseases transmission by mosquito vector (Vinogradova, 2008). The dispersal of mosquito vectors is an important factor underlying the spatial distribution of mosquitoes, and plays a major role in shaping their population structure (Service, 1997). Hence, information on mosquito survivorship, dispersal ability and spatial population genetic structure across heterogeneous landscapes is critical for understanding disease transmission risk (Carter *et al*., 2000) and planning vector monitoring and control methods (Killeen *et al.*, 2003; Hoesterey, 2011). Molecular studies of pest organisms have focused on populations over large geographic scales (Sharma *et al.*, 2010) and on local landscape features at country or city levels (Fonseca *et al.*, 2010). These studies are critical for delineating putative population boundaries, as well as assessing dispersal and gene flow across the landscape (Hoesterey, 2011). A link landscape genetic approach to classical population genetics provides a powerful framework for studying spatial distribution of genetic variation and spatial patterns of population connectivity (Manel *et al.*, 2003; Storfer *et al.*, 2010).

At the regional spatial scale genetic differentiation among populations is supposed to be caused by restricted or absent migration between fragments (Broaut *et al*., 2003). In continuous habitats, genetic differentiation can also be present (Ehrich and Stenseth, 2001). Local genetic drift associated with habitat preference and dispersal between neighbouring areas may create genetic structure, a process called "isolation by distance" (IBD) (Wright, 1943). In such continuous populations with spatially limited dispersal, levels of gene flow tend to decrease with increasing geographic distances, which results in increasing genetic differentiation among individuals (Broquet *et al.*, 2006). IBD model can be tested by analysing the distribution of pairwise estimates of genetic distances between individuals (Rousset, 2000). Still, IBD pattern reflects limitations of species-specific dispersal and gene flow that are independent of any specific landscape features (Balkenhol *et al.*, 2009). Hence, insights from landscape genetics shed light on connectivity of natural populations from both undisturbed (insecticide untreated) and anthropogenically (insecticide treated) altered landscapes (Rašić, 2011).

Divergent selection, which can arise between sympatric forms occupying separate niches, influences the evolution of reproductive barriers, and ecological speciation or isolation by ecology (Thibert-Plante and Hendry, 2010; Wolf *et al.*, 2010). However, diverged populations might be connected by gene flow at the early stages of differentiation/speciation. During the early period of ecological differentiation most of the genome, at least initially, might be subject to the homogenizing effects of gene flow (Nosil *et al.*, 2009; Butlin, 2010), but genomic regions that directly contribute to local adaptation should be highly genetically diverged (White *et al.*, 2010). Consequently, alleles at neutral markers unlinked to selected loci might be exchanged between populations from different habitats (Thibert-Plant and Hendry, 2009).

Insect species, especially mosquitoes and other public-health pests, have experienced a powerful agent of selection by insecticides (Chevillon *et al.*, 1995). To study local dispersion patterns across treated and non-treated areas, genes such as hexokinase (HK) with polymorphism independent of insecticide use (Chevillon *et al.*, 1995) provide important information for vector control strategies. Hexokinases (EC2.7.1.1) are enzymes which occur in all eukaryotic and prokaryotic cells as the first step in the utilization of glucose (Knutsen *et al.*, 1969; Kulkarni *et al.*, 2002). It is an important regulatory enzyme as it indicates phosphorylation of glucose via glycolysis and pentose phosphate pathway and activates the formation of glycogen and complex carbohydrates from glucose (Smyth, 1994).

The members of the *Culex pipiens* complex are of great medical and veterinary importance as vectors for various bacterial (Vinogradova, 2000), filarial and viral pathogens (Becker *et al.*, 2010). Since West Nile virus outbreaks spread rapidly in the USA and in parts of Europe, the zoogeography, taxonomy and identification of *Cx. pipiens s.l.* and related taxa have received high attention (Cornel *et al.*, 2003).

The *Cx. pipiens* subgroup (Sirivanakarn, 1976) consists of species *Culex pipiens* L., *Cx. quinquefasciatus* Say, *Cx. australicus* Dobrotworsky et Drummond and *Cx. globocoxitus* Dobrotworsky (http://wrbu.si.edu). The *Cx. pipiens* complex includes first three species, while subspecies *Cx. pallens* Coquillett and *Cx. pipiens* are belonged to *Cx. pipiens s.l.* (http://mosquito-taxonomic-inventory.info). However, taxonomic status of members of the *Cx. pipiens* complex is a still matter of discussion. Although introgression in non-indigenous areas between *pipiens* and *quinquefasciatus* was registered, these taxa have been recognized as independent species cohesion (Harbach, 2012). On the other side, taxonomic status of *pipiens* and *molestus* of *Cx. pipiens* has been a subject of debate, ranging from distinct species (Miles and Paterson, 1979; Weitzel *et al*,. 2009) to physiological forms with considerable genetic introgression (Harbach *et al.*, 1984; Chevillon *et al.*, 1998; Gomes *et al.*, 2009; Farajollahi *et al.*, 2011). Biologically, ecologically and genetically different properties of *molestus* and *pipiens* forms are likely resulted by complex of evolutionary processes operating at spatial and temporal scales. Hence, diagnostic phenotypic traits modulated by environmental factors cannot be fully reliable in discrimination *Cx. pipiens* ecotypes (Chevillon *et al*., 1998). Since expression of the phenotypes depends on genetic-environmental interaction, different selection pressures operated in divergent climatic regions of this widespread species would favour distinction in diagnostic value of the traits. For instance, habitat-dependent differentiation at the *Aat*-1 locus found in the *Cx. pipiens* ecotypes from London area (Byrne and Nichols, 1999) is not registered in samples from Southern Europe (Chevillon *et al*., 1998). In addition, focus on the diagnostic value of microsatellite loci has generated two approaches. The first sees microsatellite loci as useful marker for delimitating *pipiens* (anautogeneous) from *molestus* (autogeneus) ecotypes (Fonseca *et al*., 2004; Gomes *et al*., 2009; 2013; Kothera *et al*., 2009; 2013; Strickman and Fonseca, 2012). Contrary to this, Danabalan *et al*. (2012) registered misidentification between *Cx. pipiens s.l.* and *Cx. torrentium* Martini where there are sympatric in the United Kingdom. However, barcode fragment of the mitochondrial cytochrome oxidase I (5' end COI mtDNA) has been proposed to be robustness molecular marker in defining their taxonomic status (Danabalan *et al*., 2012).

The taxonomic, phenotypic, ecological, physiological and genetical extensive diversity of *Cx. pipiens* motivated us to study dispersion pattern of the "urban" and "rural" samples. This study, as a part of a larger research of genetic and phenotypic variation of *Cx. pipiens* (Krtinić, unpublished data; Krtinić *et al*., 2012) focused on the genetic diversity at the HK loci. The aim of this study was therefore to determine spatial variation at HK isozyme loci in population of the *Cx. pipiens* samples across the southern part of Pannonia Plain of Central Europe (testing Isolation by distance model), and at fine-scale level, between ecologically different larval habitats (testing Isolation by ecology model). In this study we used samples previously morphologically studied, when "urban" and "rural" samples were distinguished by parameters of the larval respiratory siphon [siphonal index (Krtinić *et al*., 2012), pecten teeth (Krtinic, unpublished data)] and adult traits [wing size and shape (Krtinic, unpublished data)]. We wish to estimate spatial diversity at HK isozyme loci in a landscape genetic study comparing landscape pattern of "urban" (II) and "rural" (III) samples. Since HK provide valuable information on *Cx. pipiens* dispersion pattern, the findings were discussed in relation to their significance for the management of vector-control strategies.

Materials and methods

Sample collection

Larvae of *Cx. pipiens* were collected from a broader area of the city of Novi Sad (45°15'N 19°50'E) during the period from June 2009 to June 2010 from three different biotypes: 1. street manholes (the underground habitat of urban type; SIa) and basements (the underground habitat of urban type; SIb), 2. draining ditches and ponds in the city (above ground habitat of urban type; SII) and 3. ponds (swamps) outside urban area (above ground habitat of rural type; SIII), and from another seven regions on the Autonomous Province of Vojvodina (northern part of Serbia) during July and August 2009 from two habitat types: 1. Above ground habitat of urban type; SII and 2. Above ground habitat of rural type; SIII (figure 1, table 1). Larvae of SIII in Novi Sad were collected in Special Nature Reserve "Koviljsko-Petrovaradinski rit" with a complex of marshes and forest ecosystems (in 2004 it was included in the list of important water-related protected areas in the Danube basin - ICPDR, since 2005 this wetland is IPA - Important Plant area, and also a Ramsar Site candidate).

Samples were collected along Vojvodina crossing intensively treated and sporadically treated areas. In Novi Sad insecticide such as organophosphates, pyrethroids, insect-growth regulators and biological agents have

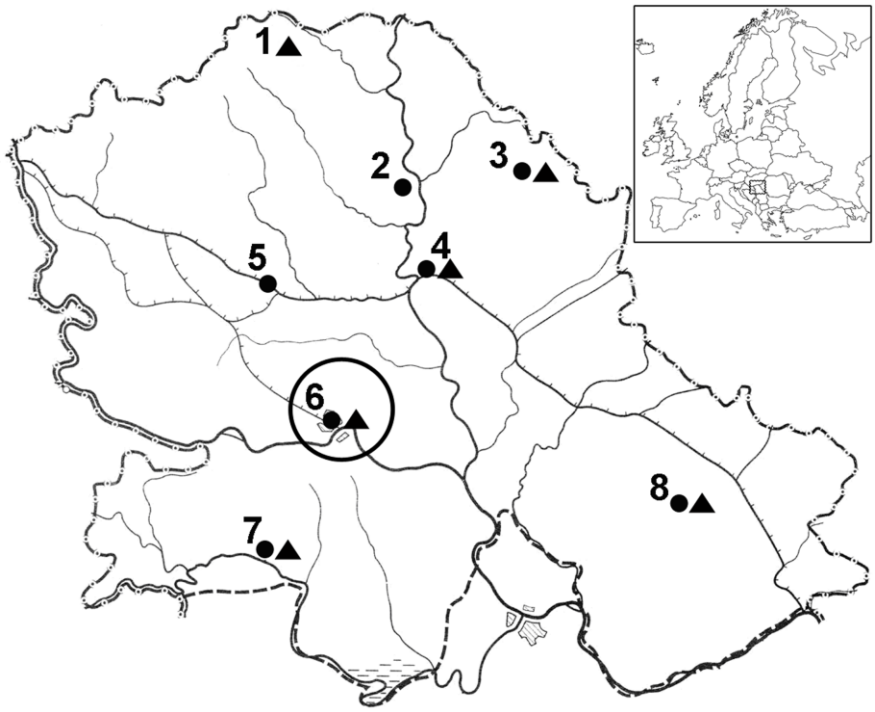

Figure 1. Map of the Autonomous Province of Vojvodina. Origin of the analyzed populations of *Cx. pipiens*: 1. Subotica (46°07'N 19°40'E) (SU); 2. Ada (45°48'N 20°08'E) (AD); 3. Kikinda (45°49'N 20°28'E) (KI); 4. Novi Bečej (45°36'N 20°08'E) (NB); 5. Vrbas (45°34'N 19°38'E) (VR); 6. Novi Sad (45°15'N 19°50'E) (NS, regularly treated area); 7. Sremska Mitrovica (44°59'N 19°51'E) (SM); 8. Alibunar (45°04'N; 20°57'E) (AL). SII-filled circle; SIII- filled triangle. Novi Sad is an intensively insecticide treated area (in circle).

Table 1. Population code, ecotypes of samples and number of collected specimens.

	Novi Sad	Vojvodina
SI - the underground habitat of urban type	68	-
SIa - street manholes	24	-
SIb -basements	44	-
SII - aboveground habitat of urban type (draining ditches and ponds in the city)	57	119
SIII - aboveground habitat of rural type (ponds -swamps) outside urban area	23	76

been applied intensively in both urban and rural areas since the 1970s and early 1980s. Across Vojvodina, mosquito control activities were conducting occasionally (not every year and less intense during the season) mainly limited on urban areas.

Sampled larvae were reared separately in the laboratory in original water without feeding to adults and then stored at −20 °C until used for enzyme characterization. Specimens were identified based on the morphological characters of adults, defined for *Cx. pipiens* (Becker *et al.*, 2010).

Allozyme analysis

Allozyme variability of hexokinase (2.7.1.1. HK; *Hk*-2, *Hk*-3, *Hk*-4) were analyzed using 5% polyacrylamide gel electrophoresis (Munstermann, 1979). Duration of electrophoretic run at 90 mA (135-220 V) was 2.5-3 hr. Enzymes were extracted from whole body in Tris-Boric-EDTA (pH 8.9) buffer and D-glucose as substrate; homogenates were centrifuged at 15000 rpm for 5 min at 4 °C.

The electrophoresis of individuals from different populations was conducted in the same gel for direct intra- and interpopulation comparison. Genotype and allele frequencies were calculated directly from the observed banding patterns based on the genetic interpretation of zymograms. Loci and alleles were numbered with respect to order of increasing anodal migration.

Statistical power of allozyme loci in population differentiation

Allelic frequencies were used for analyses of subpopulation genetic differentiation in *Cx. pipiens*. Statistical analyses of allelic and genotypic frequencies were performed using the computer program BIOSYS-2 (Swofford and Selander, 1989). To assess if the sampling and the HK alleles used was enough to detect a structure if there is one (i.e. power of the tests) we conducted several simulations using the computer program POWSIM version 4.1 (Ryman and Palm, 2006). The program detects significant differentiation (using χ^2 and Fisher's exact tests) under a specified level of popula-

tion divergence given by $1 - (1 - 1/2Ne)^t$, where t is the time since divergence and Ne is the effective population size. We simulated scenarios assuming different sample sizes, levels of divergence, with populations being separated for 250 generations, each with Ne of 5000. The values were used to simulate a case where the split of populations are very recent, and the populations are very large. Hence, this is a very conservative test of power, as if time is longer and Ne is smaller, it would be much easier to find significant results. Significance estimates were based on 1000 independent simulations.

We used the program STRUCTURE (Pritchard *et al.*, 2000) to infer genetic population structure at both ecotypes and individual levels using genotypes from *Hk*-2, *Hk*-3 and *Hk*-4 loci of 148 individuals from Novi Sad and 195 individuals from Vojvodina. All individuals were combined into one dataset for analysis, without any *a priori* population assignments and admixture was allowed with a single value of Δ inferred for all populations. We evaluated K values, the number of assumed populations, from 1-3 using a burn-in of 10000 and 10000 MCMC (Markov chain Monte Carlo) for each value of K. Each value of K was run five times to evaluate stability. The model choice criterion implemented in structure to detect the true K is an estimate of the posterior probability of the data for a given K, $\Pr(X|K)$ (Pritchard *et al.*, 2000). This value, called 'Ln P(D)' in structure output, is obtained by first computing the log likelihood of the data at each step of the MCMC. Then the average of these values is computed and half their variance is subtracted to the mean. This gives 'Ln P(D)', the model choice criterion to which we refer as L (K) afterwards. True number of populations (K) is often identified using the maximal value of L (K) returned by structure (Pritchard *et al.*, 2000). However, we observed in our simulations that in most cases, once the real K is reached, L (K) at larger Ks plateaus or continues increasing slightly (a phenomenon mentioned in the structure's manual, Pritchard and Wen, 2003). Following Evanno *et al.* (2005), we calculated ΔK, which corresponds to the rate of change of the likelihood between successive K values, using the program STRUCTURE HARVESTER v0.6.92 (Earl and vonHoldt, 2012) to identify the best supported values of K. Still, it is important to take into consideration that estimating the most likely number of clusters needed to explain the observed data is challenging and the results may be sensitive to the number of loci used, the variation at these loci, the rate of gene flow, and the number of individuals typed (Evanno *et al.*, 2005; Huelsenbeck and Andolfatto, 2007).

Spatial genetic structure

A landscape shape interpolation of genetic distances, which interpolates observed genetic distances across the landscape and shows them graphically as heights (Z axis) in a graph where the base (values along X and Y axes) represents the geographic space, was obtained using the software Alleles in Space (AIS) (Miller, 2005). We used several different distance weighting values (a = 0.25-2), grid sizes, and both raw and residual genetic distances to make sure that interpretations were not sensitive to these parameters. Geographic distances were calculated using latitude/longitude coordinate. Across the genetic landscape, the peaks and troughs indicate high and low genetic distances between individuals respectively. Using AIS we also test for genetic barriers with Monmonier's maximum difference algorithm method (Monmonier, 1973). This procedure locates barriers to gene flow by iteratively identifying sets of contiguous, large genetic distances along a connectivity network.

We used two tests in AIS software to examine the correlation between genetic and geographic distance: a Mantel test and spatial autocorrelation. Mantel tests determine correlation between genetic and geographic distances. Spatial autocorrelation compares the average genetic distance between pairs of individuals over distance classes, and is summarized with the global statistic V. For both analyses, significance was tested using 1000 and 10000 permutations.

Results

Allozyme variation at HK loci

In samples from Novi Sad the presence of four alleles at HK loci formed four homozygote ($Hk^{100/100}$, $Hk^{102/102}$, $Hk^{104/104}$ and $Hk^{106/106}$) and one heterozygote ($Hk^{100/104}$) genotypes. The presence of rare and unique Hk^{106} allele in SIa was identified, while Hk^{102} was rare in all samples except in SIa (manholes). Study of genetic diversity at regional scale revealed in all populations from Vojvodina two homozygote ($Hk^{100/100}$, and $Hk^{104/104}$) and one heterozygote ($Hk^{100/104}$) genotype, and rare and unique homozygote $Hk^{102/102}$ only in "rural" sample in Novi Sad.

Testing Isolation-by-ecology model

A model of local differentiation according to larval habitat was evaluated for individuals from three different habitats in Novi Sad using a Bayesian model-based approach. This analysis failed to uncover any population structure based on the HK isozyme system. For three samples (SI, SII and SIII) there was no clear indication of clustering because all samples showed a high degree of admixture. In STRUCTURE analysis performed with K values from 1 to 7, average log-likelihoods across five replicate STRUCTURE runs reached a first plateau at K = 2 (figure 2) indicating that 2 is most probably number of clusters (based on declining rate of increase in $\Pr(X|K)$ as K increases rather than by the absolute maximum likelihood; Pritchard *et al.*, 2000; Evanno *et al.*, 2005). Evanno's ΔK coincide with log-likelihood estimates of structuring. However, these groupings do not suggest any overt ecological pattern since proportion of individuals classified into the two clusters (clusters 1 and 2) were almost even in all samples (figure 2A). As a result, we consider that there is an absence of genetic discontinuity of *Cx. pipiens* samples (figure 2A). Likewise, for separated samples SI, SIa and SIb (manholes and basements) all genetic clusters were almost evenly represented in samples (figure 2B) and assignment indexes were close to $1/K$. To test a statistical

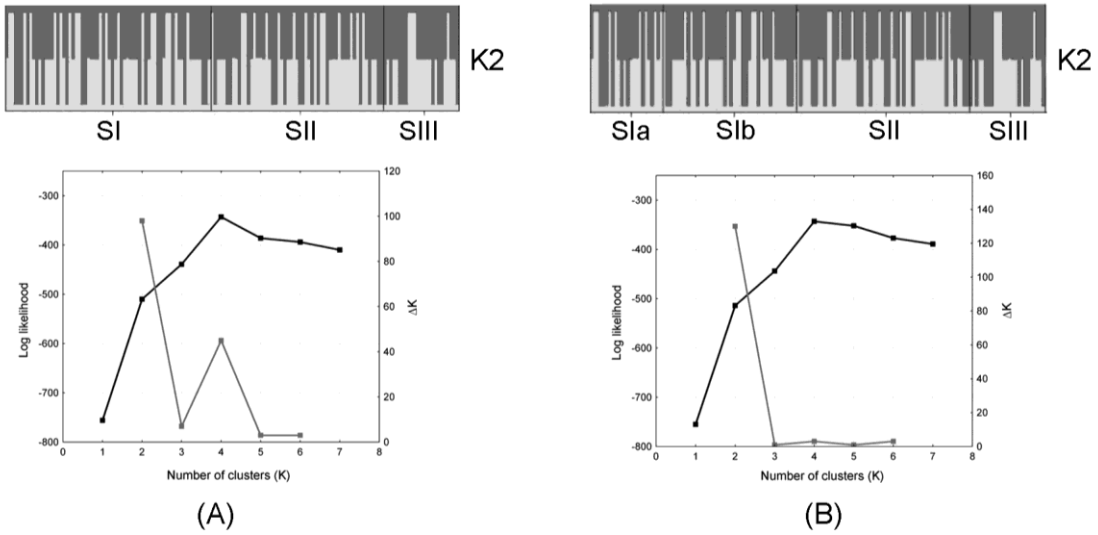

Figure 2. Membership of *Cx. pipiens* from Novi Sad individuals to a number of presumed "populations". A) SI, SII, SIII; B) SI divided in: SIa (manholes) and SIb (basements). Population clusters ($K = 2$) determined by the a priori Bayesian cluster method in STRUCTURE. Each vertical line represents an individual's probability of belonging to one of K clusters (represented by different colours). Estimates of the likely number of clusters (K). Black squares show the marginal log likelihoods of the data $\Pr(X|K)$ when the number of clusters (K) is fixed to different values averaged over five STRUCTURE runs. The grey squares denote ΔK, an *ad hoc* indicator of the uppermost hierarchical level of structure detected, based on the rate of change in $\Pr(X|K)$ between successive K-values.

power of allozyme data we did power simulations conducted on samples from three larval microhabits from Novi Sad. POWSIM indicated that given the polymorphism of loci and the sample sizes used in the study, the probability of detecting F_{ST} values as low as 0.025 was 80%. The detection probability of F_{ST} as low as 0.008 still resulted in a reasonable probability, above 50%, to detect a true F_{ST} this small. Thus, we are confident that, given the variability of loci and sampling effort, we can find differences if they exist.

Ecological model of isolation also was tested for populations of *Cx. pipiens* from Vojvodina. Genetic clustering analysis revealed no resolution between "urban" (SII) and "rural" (SIII) samples (figure 3). In each group, all individuals of both samples were almost evenly represented suggesting an absence of differentiation based on HK isozyme system. With value of $K = 2$, assignment indexes were almost the same and close to $1/K$ for all "urban"/"rural" pairs, except for Novi Bečej (NB) individuals of the SIII sample where more than 67% individuals were in one of two assumed clusters.

Testing Isolation-by-distance model

The model of differentiation due to local isolation was employed to test for the possibility of reduced gene flow between distant populations among "urban" and "rural" samples from Vojvodina. In STRUCTURE analysis performed with K values from 1 to 7, average log-likelihoods across five replicate STRUCTURE runs reached a first plateau at $K = 2$ (figure 4A) for "urban" ecotype, indicating that 2 is the most probably number of clusters. Evanno's ΔK coincided with log-likelihood estimates of structuring. However, these groupings do not suggest any overt geographical pattern, because the proportion of individuals classified into two clusters (clusters 1 and 2) were almost even in all samples (figure 4A). As a result, we consider that there is an absence of genetic discontinuities of the *Cx. pipiens* samples (figure 4A). Although for the "rural" sample the estimated likely number of clusters is $K = 3$, (figure 4B), genetic clusters were almost evenly represented in populations (figure 4B). The exception was AL population with over 45% of individuals classified in one cluster (represented by black) unlike the other "rural" populations mostly classified in two other clusters, which is in concordant with Monmonier's algorithm implemented by Alleles in Space (see results below). It should be noted that these are very slight differences suggesting genetic homogeneity of *Cx. pipiens* in Vojvodina region based on HK isozyme system. To analyse the statistical power for detecting differentiation we used POWSIM on SII samples across Vojvodina province. Simulation results for detecting differentiation among SII samples showed 60% for both χ^2 and Fisher's exact test where Ne/t combinations corresponded to 5000/250. Therefore, the power analysis revealed that HK loci were sufficient to provide a 60% probability of detecting an F_{ST} of 0.025 when analyzing the SII data. In addition, POWSIM simulations with SIII data suggest an average probability of 60% to detect a true differentiation of $F_{ST} = 0.014$ resulting from both the χ^2 and Fisher's exact tests.

Further, we tested correlation between genetic and geographic distances for "urban" (SII) and "rural" (SIII) populations from Vojvodina separately. There was no evidence of isolation by distance for isolates with recorded geographic locations based on both the Mantel test (SII- $r = -0.0159$, $P = 0.694$; SIII- $r = 0.0304$, $P = 0.210$) and spatial autocorrelation (SII- $V = 0.0257$, $P = 0.251$; SIII- $V = 0.0385$, $P = 0.297$).

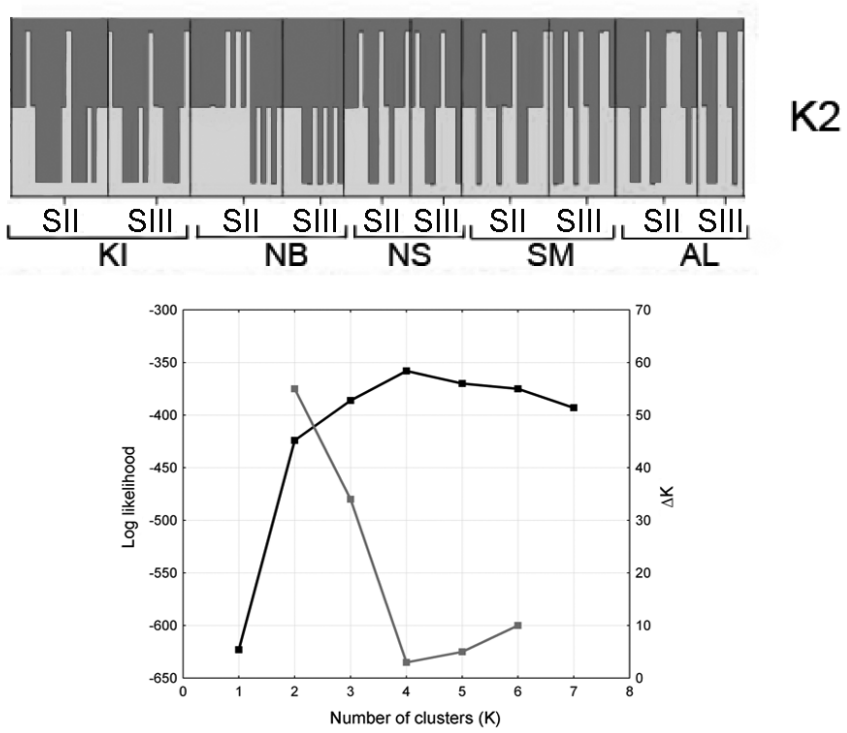

Figure 3. Membership of *Cx. pipiens* from Vojvodina individuals to a number of presumed samples, SII and SIII. Population clusters (K = 2) determined by the a priori Bayesian cluster method in STRUCTURE. Each vertical line represents an individual's probability of belonging to one of K clusters (represented by different colors). Estimates of the likely number of clusters (K). Black squares show the marginal log likelihoods of the data Pr(X|K) when the number of clusters (K) is fixed to different values averaged over five STRUCTURE runs. The grey squares denote ΔK, an *ad hoc* indicator of the uppermost hierarchical level of structure detected, based on the rate of change in Pr(X|K) between successive K-values.

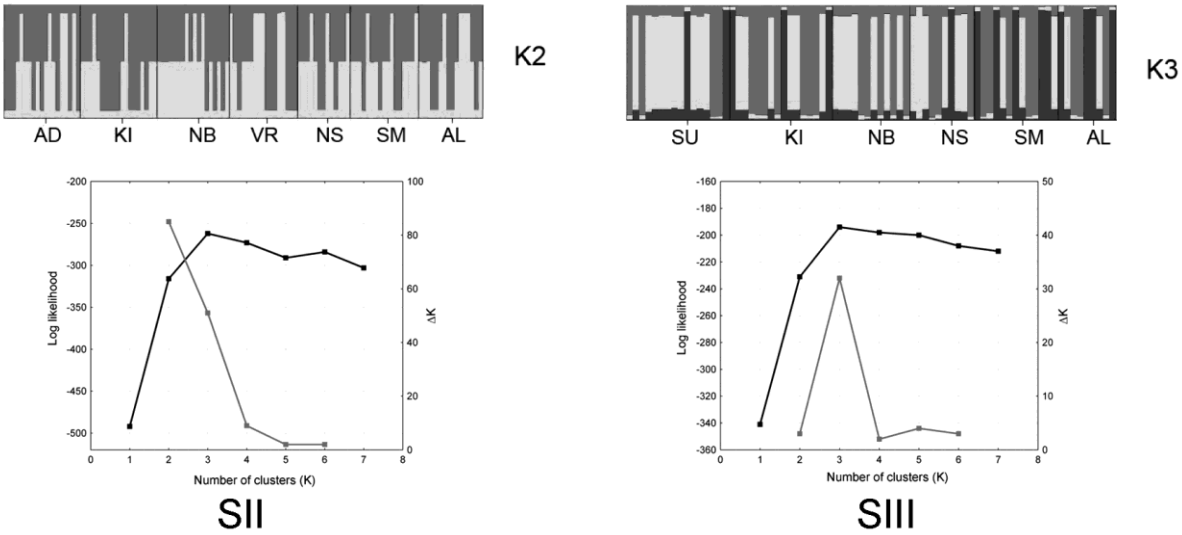

Figure 4. Membership of *Cx. pipiens* from Vojvodina individuals to a number of presumed samples, SII and SIII. Population clusters (K = 2; K=3) determined by the a priori Bayesian cluster method in STRUCTURE. Each vertical line represents an individual's probability of belonging to one of K clusters (represented by different colors). Estimates of the likely number of clusters (K). Black squares show the marginal log likelihoods of the data Pr(X|K) when the number of clusters (K) is fixed to different values averaged over five STRUCTURE runs. The grey squares denote ΔK, an *ad hoc* indicator of the uppermost hierarchical level of structure detected, based on the rate of change in Pr(X|K) between successive K-values.

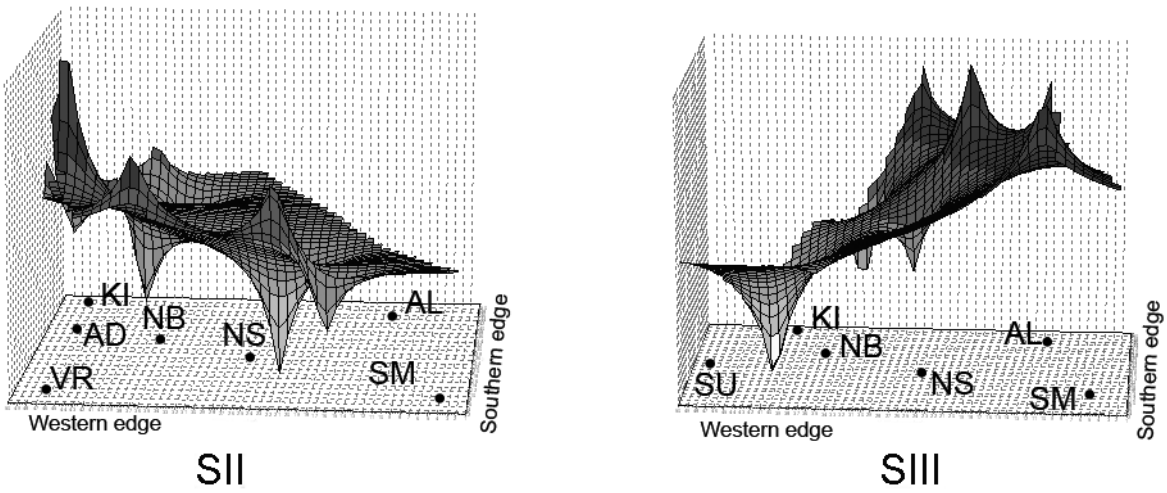

Figure 5. Results of genetic landscape shape interpolation analysis for populations of *Cx. pipiens* using a distance weighting parameter (*a*) of 1 and raw genetic distances. *X* and *Y* axes correspond to geographic locations and surface plot heights reflect genetic distances. Qualitatively similar results were obtained using both raw and residual genetic distances, different grid sizes, and a range of distance weighting parameters. The positions of sampling locations in the "base" of the graph are approximate.

Using individual spatial information, we could identify fine-scale genetic structure of both "urban" and "rural" samples by genetic landscape shape analysis that allows the graphical representation of genetic distance pattern across landscape through interpolation procedures generated in AIS. This analysis based on HK isozyme system revealed genetic distances associated with slightly increased inter-individual genetic distance. For "urban" sample it was peaked in the western part of the study region (Vrbas, VR population), while for "rural" sample eastern part (Alibunar, AL population) was separated (figure 5), suggesting partially restricted gene flow. The same general pattern was observed regardless of the choice of grid size and distance weighting parameter. In addition, Monmonier's maximum difference algorithm identified potential barriers to *Cx. pipiens* migration which coincide with results of landscape shape interpolations of genetic distances.

Discussion

Regional and fine-scale pattern of variation at HK loci

Results presented herein did not support any genetic structuring at regional- and at fine-spatial scale in *Cx. pipiens*. By testing congruence genetic differentiation with larval habitat differences (the ecological model) we evaluated genetic admixture among samples originating from street manhole - the underground habitat of urban type (SI), drainage ditch and pond in the city - aboveground habitat of urban type (SII), and pond (swamp) outside urban area - aboveground habitat of rural type (SIII). Similarly, a lack of correlation between genetic and geographic distances (tested by Mantel test and spatial autocorrelation) among "urban" (SII) and "rural" (SIII) samples across the Vojvodina region was estimated.

Rejecting both isolation by distance and isolation by ecology model the findings imply that there is a high gene flow between individuals sampled throughout urban and rural environments. Thus, the absence of identifiable genetic clusters suggests that genetic homogeneity characterized *Cx. pipiens* from southern part of the Pannonia. Indeed, some previous studies have shown that *Cx. pipiens* requires a considerable distance to display IBD (Cui *et al.*, 2007). Similar to our results, Chevillon *et al.* (1995) using genes with a polymorphism independent of insecticide selection pressure (such as *Aat*-1, *Aat*-2, *Pgm*, *Gpi* and *Hk*) estimated high gene flow between populations over the whole range of the study (in this case, it was 850 km). A lack of correlation between genetic and geographic distance (IBD) suggested that migration is counteracting the effect of drift and local selection regimes of the populations (Chevillon *et al.*, 1998). Genetic homogeneity observed in samples across Vojvodina imply that populations of *Cx. pipiens* are not distributed according to "stepping stone model" (Wright, 1943), but under Wright's island model of migration (1931; 1943).

Furthermore, in addition to ecological factors, human factors such as insecticide application have an influence on genetic structure (Paupy *et al.*, 2005). Insecticide treatments as stress on mosquito populations can have an effect on genetic diversity (Chevillon *et al.*, 1995; Paupy *et al.*, 2005). Still, it has been observed that decrease in genetic diversity occurs only after strong bottleneck and in relatively isolated populations (Hoffmann and Willi, 2008). It has been suggested that vector control efforts could impose severe genetic bottlenecks on local populations (Bosio *et al.*, 2005). In this study, comparing less and sporadically treated areas in Vojvodina, decrease in genetic variability in *Cx. pipiens* was not observed from insecticide treated sites in Novi Sad. Moreover, "rural" populations experienced severe bottlenecks during winter, which might influence genetic diversity of the sub-

sequent populations. Mosquito populations experience different demographic histories due to use temporary ponds for their reproduction as well (Krtinić, field observation). Because of wintering bottleneck of "rural" sample, we compared HK genotypes of individuals from both ecotypes at the same area (KI, NB, NS, SM, AL; figure 3). Absence of genotypic portioning at hexokinase was estimated between individuals of different biological properties as well. Thus, our findings suggest that populations after bottlenecks (influenced by insecticide treatment and/or overwinter decrease in size) has been rapidly recovered and/or recolonized by migrants.

Landscape genetics provides full understanding how the spatial distribution of genetic variation arises in populations. The main focus of landscape genetics is the study of interaction between landscape features and gene flow, as well as microevolutionary processes (genetic drift, selection) (Manel *et al.*, 2003; Storfer *et al.*, 2010). Therefore, we implemented landscape genetic approach to better understand spatial distribution of genetic diversity at HK loci. Comparing genotypes of individuals belonging to particular sample (SII, SIII) across the Vojvodina region, no genetically distant sample was registered. Still, AIS analysis was provided for better insight into our understanding of gene flow and population structure of the species. This spatial analysis highlighted sample of "urban" sample from western part of Vojvodina (VR population) and individuals of "rural" sample from eastern part (AL population), being genetically differentiated from other analyzed individuals of SII and SIII, respectively. VR population is differentiated may be due to high industry pollution of canal network in the area. AL population is separated from others by Deliblato sands, an isolated complex of sand masses with a distinctly undulating dune relief on an area of over 380 km^2. Because there are no surface watercourses, the Special Nature Reserve "Deliblato sands" (International status: IBA site 1989 - Important Bird area, and also a Ramsar Site candidate) is likely a barrier to dispersal.

Implications for vector monitoring

Understanding the patterns of gene flow and population genetic structure of *Cx. pipiens* has important practical implications for the management of vector control strategies. First, the use of genes which are not affected by organic insecticide provides essential information linking to population structure and dispersal mode of the species of interest (Paris *et al.*, 2010). Our study shows that there is substantial lack of genetic structure of *Cx. pipiens* in the study area (at regional scale) and among ecologically heterogeneous habitats (at fine spatial scale). This genetic homogeneity of the populations across intensively treated and sporadically less treated areas gives better insight into the role of evolutionary mechanisms such as natural selection, genetic drift and gene flow, and past history of the population (bottleneck) in determining population structure. Given a low dispersal ability of *Cx. pipiens* (migrates usually less than 500 m in human settlements, Becker *et al.*, 2010) control measures are restricted to urban areas. Observed high genetic connectivity among populations herewith indicated that the contemporary process such as gene flow is an important factor underlying genetic structure of *Cx. pipiens*. Indeed, it was considered that gene flow is the most relevant factor for vector control management (Hlaing *et al.*, 2010).

Apart from adaptive potential of public-health pests, a spread of insecticide resistance alleles is the main problem for vector control (Gubler, 2002). Additionally, insecticide resistance may spread by gene flow throughout the urban area from the treated population (Lenormand *et al.*, 1998). Therefore, data considering dispersal trajectories of the vector species are essential for predicting the geographical spread pattern of a resistance allele (Hlaing *et al.*, 2010). Designing a sustainable vector control strategy for the species with detectable gene flow, such as *Cx. pipiens,* must remain a regional effort. As it has been previously suggested (Paupy *et al.*, 2005; Porretta *et al.*, 2007; Paris *et al.*, 2010), reliable knowledge of the population structure at the appropriate geographical scale and coordinated treatments of breeding habitats would give satisfactory control programme.

Acknowledgements

This work was supported in part by the Ministry of Science of Serbia (Dynamics of gene pool, genetic and phenotypic variability of populations, determined by the environmental changes, No. 173012), and the Provincial Secretariat for Science and Technological Development (Molecular and phenotypic diversity of taxa of economical and epidemiological importance, and endangered and endemic species in Europe). B. K. is supported by Ciklonizacija d.o.o. Novi Sad.

References

BALKENHOL N., GUGERLI F., CUSHMAN S. A., WAITS L. P., COULON A., ARNTZEN J. W., HOLDEREGGER R., WAGNER H. H., ARENS P. F. P., CAMPAGNE P., DALE V. H., NICIEZA A. G., SMULDERS M. J. M., TEDESCO E., WANG H., WASSERMAN T., 2009.- Identifying future research needs in landscape genetics: where to from here?- *Landscape Ecology*, 24: 455-463.

BECKER N., PETRIĆ D., ZGOMBA M., BOASE C., DAHL C., LANE J., KAISER A., 2010.- *Mosquitoes and their control* 2nd edition.- Springer Verlag, Berlin Heidelberg, Germany.

BOSIO C. F., HARRINGTON L. C., JONES J. W., SITHIPRASASNA R., NORRIS D. E., SCOTT T. W., 2005.- Genetic structure of *Aedes aegypti* populations in Thailand using mitochondrial DNA.- *American Journal of Tropical Medicine and Hygiene*, 72: 434-442.

BROAUT C., SENNEDOT F., AUDIOT P., LEBLOIS R., RASPLUS J. Y., 2003.- Fine-scale genetic structure of two carabid species with contrasted levels of habitat specialization.- *Molecular Ecology*, 12: 1731-1745.

BROQUET T., RAY N., PETIT E., FRYXELL J. M., BUREL F., 2006.- Genetic isolation by distance and landscape connectivity in the American marten (*Martes americana*).- *Landscape Ecology*, 21: 877-889.

BUTLIN R. K., 2010.- Population genomics and speciation.- *Genetica*, 138: 409-418.

BYRNE K., NICHOLS R. A., 1999.- *Culex pipiens* in London underground tunnels: differentiation between surface and subterranean population.- *Heredity*, 82: 7-15.

CARTER R., MENDIS K. N., ROBERTS D., 2000.- Spatial targeting of interventions against malaria.- *Bulletin of the World Health Organization*, 78: 1401-1411.

CHEVILLON C., PASTEUR N., MARQUINE M., HEYSE D., RAYMOND M., 1995.- Population structure and dynamics of selected genes in the mosquito *Culex pipiens*.- *Evolution*, 49: 997-1007.

CHEVILLON C., RIVET Y., RAYMOND M., ROUSSET F., SMOUSE P. E., PASTEUR N., 1998.- Migration/selection balance and ecotypic differentiation in the mosquito *Culex pipiens*.- *Molecular Ecology*, 7: 197-208.

CORNEL A. J., MCABEE R. D., RASGON J., STANICH M. A., SCOTT T. W., COETZEE M., 2003.- Differences in extent of genetic introgression between sympatric *Culex pipiens* and *Culex quinquefasciatus* (Diptera: Culicidae) in California and South Africa.- *Journal of Medical Entomology*, 40 (1): 36-51.

CUI F., QIAO C. L., SHEN B. C., MARQUINE M., WEILL M., RAYMOND M., 2007.- Genetic differentiation of *Culex pipiens* (Diptera: Culicidae) in China.- *Bulletin of Entomological Research*, 97: 291-297.

DANABALAN R., PONSONBY D. J., LINTON Y. M., 2012.- A critical assessment of available molecular identification tools for determining the status of *Culex pipiens* s.l. in the United Kingdom.- *Journal of the American Mosquito Control Association*, 28 (4 supplement): 68-74.

EARL D. A., VONHOLDT B. M., 2012.- STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method.- *Conservation Genetics Resources*, 4 (2): 359-361.

EHRICH D., STENSETH N. C., 2001.- Genetic structure of Siberian lemmings (*Lemmus sibiricus*) in a continuous habitat: large patches rather than isolation by distance.- *Heredity*, 86: 716-730.

EVANNO G., REGNAUT S., GOUDET J., 2005.- Detecting the number of clusters of individuals using the software structure: a simulation study.- *Molecular Ecology*, 14: 2611-2620.

FARAJOLLAHI A., FONSECA D. M., KRAMER L. D., KILPATRICK A. M., 2011.- ''Bird biting'' mosquitoes and human disease: A review of the role of *Culex pipiens* complex mosquitoes in epidemiology.- *Infection, Genetics and Evolution*, 11: 1577-1585.

FONSECA D. M., KEYGHOBADI N., MALCOLM C. A., MEHMET C., SCHAFFNER F., MOGI M., FLEISCHER R. C., WILKERSON R. C., 2004.- Emerging vectors in the *Culex pipiens* complex.- *Science*, 303: 1535-1538.

FONSECA D. M., WIDDEL A. K., HUTCHINSON M., SPICHIGER S. E., KRAMER L. D., 2010.- Fine-scale spatial and temporal population genetics of *Aedes japonicus*, a new US mosquito, reveal multiple introductions.- *Molecular Ecology*, 19: 1559-1572.

GOMES B., SOUSA C. A., NOVO M. T., FREITAS F. B., ALVES R., CÔRTE-REAL A. R., SALGUEIRO P., DONNELLY M. J., ALMEIDA A. P. G., PINTO J., 2009.- Asymmetric introgression between sympatric molestus and pipiens forms of *Culex pipiens* (Diptera: Culicidae) in the Comporta region, Portugal.- *BMC Evolutionary Biology*, 9: 262.

GOMES B., SOUSA C. A., VICENTE J. L., PINHO L., CALDERON I., AREZ E., ALMEIDA A. P. G., DONNELLY M. J., PINTO J., 2013.- Feeding patterns of molestus and pipiens forms of *Culex pipiens* (Diptera: Culicidae) in a region of high hybridization.- *Parasites & Vectors*, 6: 93.

GUBLER D. J., 2002.- The global emergence/resurgence of arboviral diseases as public health problems.- *Archives of Medical Research*, 33: 330-342.

HARBACH R. E., 2012.- *Culex pipiens*: species versus species complex- taxonomic history and perspective.- *Journal of the American Mosquito Control Association*, 28 (4 supplement): 10-23.

HARBACH R. E., HARRISON B. A., GAD A. M., 1984.- *Culex* (*Culex*) *molestus* Forskål (Diptera: Culicidae): neotype, designation, description, variation, and taxonomic status.- *Proceedings of the Entomological Society of Washington*, 86: 521-542.

HLAING T., TUN-LIN W., SOMBOON P., SOCHEAT D., SETHA T., MIN S., THAUNG S., ANYAELE O., DE SILVA B., CHANG M. S., PRAKASH A., LINTON Y., WALTON C., 2010.- Spatial genetic structure of *Aedes aegypti* mosquitoes in mainland Southeast Asia.- *Evolutionary Applications*, 3: 319-334.

HOESTEREY J. C., 2011.- Freshwater insects: bioassessment and population genetics of the tule mosquito, *Culex erythrothorax*. *Doctoral Thesis*, Faculty of San Diego State University, USA.

HOFFMANN A. A., WILLI Y., 2008.- Detecting genetic responses to environmental change.- *Nature Reviews Genetics*, 9: 421-432.

HUELSENBECK J., ANDOLFATTO P., 2007.- Inference of population structure under a dirichlet process model.- *Genetics*, 175: 1787-1802.

KILLEEN G. F., KNOLS B. G., GU W., 2003.- Taking malaria transmission out of the bottle: implications of mosquito dispersal for vector-control interventions.- *Lancet Infectious Diseases*, 3: 297-303.

KNUTSEN C., SING C. F., BREWER G. J., 1969.- Hexokinase isozyme variability in *Drosophila robusta*.- *Biochemical Genetics*, 3: 475.

KOTHERA L., ZIMMERMAN E. M., RICHARDS C. M., SAVAGE H. M., 2009.- Microsatellite characterization of subspecies and their hybrids in *Culex pipiens* complex (Diptera: Culicidae) mosquitoes along a north-south transect in the central United States.- *Journal of Medical Entomology*, 46: 236-248.

KOTHERA L., NELMS B. M., REISEN W. K., SAVAGE H. M., 2013.- Population genetic and admixture analyses of *Culex pipiens* complex (Diptera: Culicidae) populations in California, United States.- *American Journal of Tropical Medicine and Hygiene*, 46: 236-248.

KRTINIĆ B., LUDOŠKI J., MILANKOV V., 2012.- Study on siphonal measurements and usefulness in delimitation of "rural" and "urban" ecotypes of *Culex pipiens* (Diptera, Culicidae).- *Bulletin of Insectology*, 65 (1): 23-27.

KULKARNI G. V., DEOBAGKAR D. N., DEOBAGKAR D. D., 2002.- Hexokinase like protein from *Drosophila melanogaster*.- *Drosophila Information Service*, 85: 65-68.

LENORMAND T., GUILLEMAUD T., BOURGUET D., RAYMOND M., 1998.- Evaluating gene flow using selected markers: a case study.- *Genetics*, 149: 1383-1392.

MANEL S., SCHWARTZ M., LUIKART G., TABERLET P., 2003.- Landscape genetics: combining landscape ecology and population genetics.- *Trends in Ecology & Evolution*, 18: 189-197.

MILES S. J., PATERSON H. E., 1979.- Protein variation and systematics in the *Culex pipiens* group of species.- *Mosquito Systematics*, 11: 187-202.

MILLER M. P., 2005.- Alleles in space: computer software for the joint analysis of interindividual spatial and genetic information.- *Journal of Heredity*, 96: 722-724.

MONMONIER M. S., 1973.- Maximum-difference barriers: an alternative numerical regionalization method.- *Geographical Analysis*, 5 (3): 245-261.

MUNSTERMANN L. E., 1979.- Isozymes of *Aedes aegypti*: phenotypes, linkage, and use of genetic analysis of sympatric populations in East Africa. *Doctoral Thesis*, University of Notre Dame, USA.

NOSIL P., FUNK D. J., ORTIZ-BARRIENTOS D., 2009.- Divergent selection and heterogeneous genomic divergence.- *Molecular Ecology*, 18: 375-402.

PARIS M., BOYER S., BONIN A., COLLADO A., DAVID J. P., DESPRES L., 2010.- Genome scan in the mosquito *Aedes rusticus*: population structure and detection of positive selection after insecticide treatment.- *Molecular Ecology*, 19: 325-337.

PAUPY C., CHANTHA N., REYNES J. M., FAILLOUX A. B., 2005.- Factors influencing the population structure of *Aedes aegypti* from the main cities in Cambodia.- *Heredity*, 95: 144-147.

PORRETTA D., CANESTRELLI D., BELLINI R., CELLI G., URBANELLI S., 2007.- Improving insect pest management through population genetic data: a case study of the mosquito *Ochlerotatus caspius* (Pallas).- *Journal of Applied Ecology*, 44: 682-691.

PRITCHARD J. K., WEN W., 2003.- *Documentation for STRUCTURE software: Version 2*.- [online] URL: http://pritchardlab.stanford.edu

PRITCHARD J. K., STEPHENS M., DONNELLY P., 2000.- Inference of population structure using multilocus genotype data.- *Genetics*, 155: 945-959.

RASIC G., 2011.- A model system in landscape genetics - the insect inhabitants of pitchner plants. *Doctoral Thesis*, The University of Western Ontario London, Ontario, Canada.

ROUSSET F., 2000.- Genetic differentiation between individuals.- *Journal of Evolutionary Biology*, 13: 58-62.

RYMANN N., PALM S., 2006.- POWSIM: a computer program for assessing statistical power when testing for genetic differentiation.- *Molecular Ecology Notes*, 6: 600-602.

SERVICE M. W., 1997.- Mosquito (Diptera: Culicidae) dispersal - the long and short of it.- *Journal of Medical Entomology*, 34: 579-588.

SHARMA A. K., MENDKIA M. J., TIKARA S. N., KULKARNI G., VEERA V., PRAKASHA S., SHOUCHEB Y. S., PARASHARA B. D., 2010.- Molecular phylogenetic study of *Culex quinquefasciatus* mosquito from different geographical regions of India using 16S rRNA gene sequences.- *Acta Tropica*, 116: 89-94.

SIRIVANAKARN S., 1976.- Medical entomology studies - III. A revision of the subgenus *Culex* in the Oriental region (Diptera: Culicidae).- *Contibutions of the American Entomological Institute*, 12: 1-272.

SMYTH J. D., 1994.- *Introduction to animal parasitology*. 3rd Edition.- Cambridge University Press, Cambridge, UK.

STORFER A., MURPHY M. A., SPEAR S. F., HOLDEREGGER R., WAITS L. P., 2010.- Landscape genetics: where are we now?- *Molecular Ecology*, 19: 3496-3514.

STRICKMAN D., FONSECA D. M., 2012.- Autogeny in *Culex pipiens* complex mosquitoes from the San Francisco Bay area.- *American Journal of Tropical Medicine and Hygiene*, 87 (4): 719-726.

SWOFFORD D. L., SELANDER R. B., 1989.- *BIOSYS-2: a computer program for the analysis of allelic variation in genetics*.- University of Illinois at Urbana-Champaign, Urbana, USA.

THIBERTE-PLANTE X., HENDRY A. P., 2009.- Five questions on ecological speciation addressed with individual-based simulations.- *Journal of Evolutionary Biology*, 22: 109-123.

THIBERTE-PLANTE X., HENDRY A. P., 2010.- When can ecological speciation be detected with neutral loci?- *Molecular Ecology*, 19: 2301-2314.

VINOGRADOVA E. B., 2000.- Culex pipiens pipiens *mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control*.- Pensoft, Moscow, Russia.

VINOGRADOVA E. B., 2008.- Monitoring of local populations of the urban mosquito *Culex pipiens pipiens* f. *molestus* Forsk. (Diptera, Culicidae) in St. Petersburg.- *Entomological Review*, 2: 129-138.

WEITZEL T., COLLADO A., JÖST A., PIETSCH K., STORCH V., BECKER N., 2009.- Genetic differentiation of populations within the *Culex pipiens* complex and phylogeny of related species.- *Journal of American Mosquito Control Association*, 25 (1): 6-17.

WHITE B. J., CHENG C., SIMARD F., COSTANTINI C., BESANSKY N. J., 2010.- Genetic association of physically unlinked islands of genomic divergence in incipient species of *Anopheles gambiae*.- *Molecular Ecology*, 19: 925-939.

WOLF J. B. W., LINDELL J., BACKSTRÖM N., 2010.- Speciation genetics: current status and evolving approaches.- *Philosophical Transactions of the Royal Society (Biology)*, 365: 1717-1733.

WRIGHT S., 1931.- Evolution in Mendelian populations.- *Genetics*, 16: 97-159.

WRIGHT S., 1943.- Isolation by distance.- *Genetics*, 28: 139-156.

Authors' addresses: Vesna MILANKOV (corresponding author: vesna.milankov@dbe.uns.ac.rs), Ljubinka FRANCUSKI, Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia; Bosiljka KRTINIĆ, Ciklonizacija, Primorska 76, 21000 Novi Sad, Serbia.

Received April 10, 2014. Accepted September 9, 2014.