Arch. Biol. Sci., Belgrade, 61 (4), 587-598, 2009 DOI:10.2298/ABS0904587N

MOLECULAR CHARACTERIZATION OF THE HONEYBEE *APIS MELLIFERA CARNICA* IN SERBIA

N. NEDIĆ[1], L. STANISAVLJEVIĆ[2], M. MLADENOVIĆ[1], and JELENA STANISAVLJEVIĆ[2]

[1]*Faculty of Agriculture, University of Belgrade,* 11080 Belgrade, Serbia
[2]*Faculty of Biology, University of Belgrade,* 11000 Belgrade, Serbia

Abstract — The sequences COI-COII of the mitochondrial DNA region in honeybee from four geographically distant regions in Serbia (Vršac, Knjaževac, Kraljevo, and Vranje) are analyzed. The research was conducted on eight different, previously selected honeybee lines preserved (linear selection) in the four reprocenters for queen bees. All four studied honeybee lines differ in morphological and productive traits, each being specific for the corresponding region. In addition to analysis of the mtDNA sequences in Serbian honeybee, a comparative analysis of the phylogenetic group of so far known C2 haplotypes was also performed. The results revealed two novel polymorphic positions in the COI-COII mtDNA region, viz., h2 at position 3474 and l2 at position 3534 (a T nucleotide deletion in both cases) in honeybees from the regions of Vranje and Knjaževac, respectively. Two novel mtDNA haplotypes in the honeybee C2 phylogenetic group, together with C2I (the new polymorphic position l2 and G-A transition at position 3587) and C2J (the new polymorphic position h2), are described. Also, comparative analysis performed on sequences from GenBank data showed a high degree of similarity (similarity index = 99.4%) between the novel C2I mtDNA haplotype and an *A. m. cypria* haplotype originating from Turkey. Certain domestic Kranjska honeybee populations from Serbia represent an autochthonous gene pool that can be of great importance for further presentation of honeybee biodiversity. The present paper contributes to characterization of mtDNA in honeybee of Serbia.

Key words: Honeybee, *Apis mellifera,* COI-COII intergenic region, genetic variation, C2 phylogenetic group, Serbia

UDC 595.79:638.12(497.11):575

INTRODUCTION

Apis mellifera L. is an autochthonous species in Europe, Africa, and Asia (including Saudi Arabia, Iran, and the Ural Mountains in Russia). Today, this species is widely disseminated in the world due to multiple migrations and introductions. *Apis mellifera* has about 25 subspecies in different regions of the world (Ruttner, 1988; Sheppard et al., 1997). On the basis of morphological characteristics, these subspecies are classified into four main groups: C (the Carnica group, including *A. m. carnica* and *A. m. ligustica*); M (Nothern and Western European honeybees, including *A. m. mellifera, A. m. iberica*, and *A. m. intermissa*); A (the African group, including *A. m. scutellata*, *A. m. capensis*, *A. m. lamarckii*, *A. m. litorea*, *A. m. adansonii*, and *A. m. unicolor*); and O (the Oriental group, including *A. m. anatolica*, *A. m. caucasica*, *A. m. syriaca*, *A. m. pomonella*, and *A. m. cypria*) (Ruttner, 1992). Molecular data on nuclear and mitochondrial DNA (mtDNA) sequences indicate that all the subspecies are very divergent genetically. Each has its own specific behavioral and morphometric characteristics, although they can be intermixed since they belong to the same species.

Apis m. carnica belongs to the North Mediterranean C group according to the morphometrical analyses of Ruttner (1988). However, it is now known that morphological traits are not reliable enough and sufficient in studies dealing with spreading or for reconstruction of phylogenetic relations because they are sensitive to selection impact and changes in the environment. For that reason, mtDNA is being used increasingly in these analyses as an additional and more reliable genetic marker (Avise et al., 1987; Franck et al., 2000a). Analysis of mtDNA sequences is a relatively easy and simple way to reconstruct

phylogeny, which is desirable in drawing conclusions about relations among populations and the history of migrations (spreading) (Palmer et al., 2000). The variability of mtDNA can be used for both identification and phylogenetic analysis of honeybee subspecies (Smith and Brown, 1988). The honeybee mitochondrial genome can afford numerous data for phylogenetic studies on *A. mellifera*, as is evident from the great number of research papers published to date (Smith et al., 1991; Garnery et al., 1992; Meixner et al., 1993, 2000; Moritz et al., 1994; Arias and Sheppard, 1996; Sheppard et al., 1996; Franck et al., 1998, 2000a, 2000b; Palmer et al., 2000; De la Rúa et al., 2001a, 2001b, 2006; Bouga et al., 2005; Kandemir et al., 2006a, 2006b, Solorzano et al., 2009).

Based on analysis of the highly variable COI-COII mtDNA region in honeybee, five evolutionary groups have been described (Smith and Brown, 1988; Cornuet et al., 1991; Garnery et al., 1992; Arias and Sheppard, 1996; Franck et al., 2000a, 2001; Palmer et al., 2000). According to this classification, *A. m. carnica* belongs to the East Mediterranean mitochondrial line C (the C mtDNA line). It is important to state that through introduction of honey bee queen bees originating from other mtDNA lines, there can occur a hybridization with autochthonous mtDNA lines, which can later result in modification of the area of spreading of mtDNA haplotypes within the already existing genetic pool among local bees (Bouga et al., 2005).

Variability in the COI-COII mtDNA region results from differences in length (presence/absence of a P sequence, number of repeated Q sequences, possibility of small deletions) and nucleotide change. Characteristic of the mtDNA C line is that it has the shortest haplotype, with no P sequence and only one Q sequence (Cornuet et al., 1991). A relatively short sequence and absence of variation in the COI-COII mtDNA region provides a diminished potential for establishing so-called patterned variability in this line (Garnery et al., 1993). So far, within the mtDNA C group 38 haplotypes have been described: firstly, C1 (NCBI GenBank accession number: FJ478010) in *A. m. ligustica*; C2A in *A. m. carnica* and C2B in *A. m. caucasica* (Franck et al., 2000b); C2D in *A. m. macedonica* and C2C in *A. m. carnica* in Slovenia and Croatia (Sušnik et al., 2004); and recently C2E in *A. m. carnica* in Serbia (Kozmus et al., 2007). All of the mentioned haplotypes differ in only eight polymorphic positions (two insertions/deletions and six transitions) (Franck et al., 2000b; Sušnik et al., 2004; Kozmus et al., 2007). The difference between the C1 and C2 groups of haplotypes is a single cytosine base insertion in C1 at position 3428 (Franck et al., 2000a). According to the data of Solorzano et al. (2009), investigators in Turkey found a total of 11 new mitotypes (mitotype = mtDNA haplotype) from the "C" phylogenetic group of *A. mellifera* (assigned as C11, C12, C14, C15, C17, C18, C19, C20, C21, C22, and C24, their accession numbers in GenBank being: FJ037776, FJ037777, FJ037778, FJ037779, FJ037780, FJ037781, FJ037782, FJ037783, FJ037784, FJ037785, and FJ037786, respectively). The same authors state that one more haplotype assigned by them as 13, matches completely with the previously published *TrDra-3* of Kandemir et al. (2006a), which in GenBank is listed under the accession number AY618915. Solorzano et al. (2009) did not follow the usual practice in assigning haplotype nomenclature. All haplotypes mentioned in this research on Turkish honeybees differed in a total of 15 polymorphic nucleotide positions (Solorzano et al., 2009). Besides these published results, data on 11 more haplotypes established on the basis of sequences of the COI-COII mtDNA intergenic region that belong to the "C" phylogenetic group from Turkey are deposited in the NCBI GenBank. Sequences from this study were deposited in GenBank as accession numbers FJ357798 and C1a, FJ357799 and C1b, FJ357800 and C1c, FJ357801 and C1d, FJ357802 and C1e, FJ357803 and C1f, FJ357804 and C1g, and FJ357805 and C2a (*A. m. anatolica*); FJ357806 and C2f, and FJ357807 and C2g (*A. m. meda*); and FJ357808 and C2h (*A. m. caucasica*) (Özdil et al., 2009). One mitotype from Brazil is deposited in GenBank as accession number EF033655 and assigned as C1. Eight sequences of the same *A. mellifera* COI-COII mtDNA intergenic region belonging to the phylogenetic „C" group are also deposited in the GenBank basis as accession numbers: AY618912, AY618913, AY618914, AY618915 (*A. m. anatolica*), AY618921 (*A. m. cypria*), AY618916, AY618917, and AY618918

(*A. m. syriaca*) (Kandemir et al., 2006a). However, these authors also did not follow the established rules of assigning haplotype nomenclature. It is evident that they did not take into account several previously published sequences in GenBank or had no insight into the papers of authors who made a corresponding assignment before them. For this reason, the revision of these sequences would be much appreciated, both in order to avoid synonymity and to introduce new names for them.

Serbia is situated in the middle of the area of spreading of the Mediterranean evolutionary group C. The only molecular research on honeybee in this region was performed by Kozmus et al. (2007). In this research, domestic (indigenous) honeybees from three regions of Serbia were studied, and one new haplotype (C2E) was described. Sampled from previously established "type" localities, the honeybees belonged to three ecotypes (Banatski, Sjeničko-Pešterski, and Timočki) and were described on the basis of their morphometric (Stevanović, 2002) and chromosomal (Stanimirović et al., 2005) features.

The honeybee samples for this research paper were analyzed in the same laboratory (in Slovenia) immediately after samples were analyzed there by Kozmus et al. (2007), and our work thus represents a kind of continuation in the molecular characterization of Serbian honeybee populations.

The principal aim of this research was to complete the picture of variability between previously selected honeybee lines in Serbia and determine whether the observed phenotypic variability (obtained by comparative morphometric research) is matched on the molecular level as well, or whether there are some differences. Another aim was to determine the genetic and phylogeographic structural (spatial) limits of the Serbian honeybee population on the basis of data supplied by COI-COII mtDNA sequences and ascertain if there exist some genetically specific populations that could be used in development of a strategy for genetic conservation of the species *A. mellifera*.

The conservation of local populations of honeybees and their original genetic structure is a major priority in many countries. A better insight into the genetic variability of Serbian *A. mellifera* will contribute to the global task of conservation of autochthonous species.

In phylogenetic studies on honeybee populations, especially those in the Mediterranean any possible historical influence should be taken into account as well. Thus, for example, Turkish rule over almost the entire Balkan Peninsula (at the time of the Ottoman Empire) and the whole of Serbia as a central and transit country in this region in the past probably influenced the present state of honeybee genetic variability. A similar conclusion was drawn about the *A. m. ligustica* honeybee population of Sicily (Italy), into which different genetic characteristics originating from the African (A) branch and/or the Oriental (O) branch have been incorporated (Ruttner, 1988; Franck et al., 2000b).

MATERIAL AND METHODS

The honeybee samples from which mtDNA was taken and analyzed for this research were collected between May 15 and June 10, 2007 from four geographically distant reprocenters in Serbia (Nedić et al., 2007): one selected line from the region of Vršac (Vršac = 1); two lines from the region of Knjaževac (Lasovo = 2 and Stogazovac = 3); two lines from the region of Kraljevo (Baljevac = 4 and Žitniće = 5); and three lines from the region of Vranje (Kunovo = 6, Katun = 7, and Sebrat = 8) (Fig. 1). A total of eight honeybee colonies were sampled (one beehive from each selected line). The honeybees were taken directly from honeycombs in order to avoid the influence of other bees in neighboring honeybee colonies. Each honeybee was immersed in absolute ethyl alcohol (etanol) and kept at -20°C until used for laboratory analyses.

Total DNA was extracted from worker bee legs. Each sample was collected in form of a fine powder using liquid nitrogen. For extraction of DNA, we used the JETQUICK Tissue DNA Spin Kit (Genomed) with a slight modification of the protocol described by Bowtell (1987). The concentration of DNA was measured with the aid of a fluorometer. A concentration of 100 ng/μl was used for further DNA analysis.

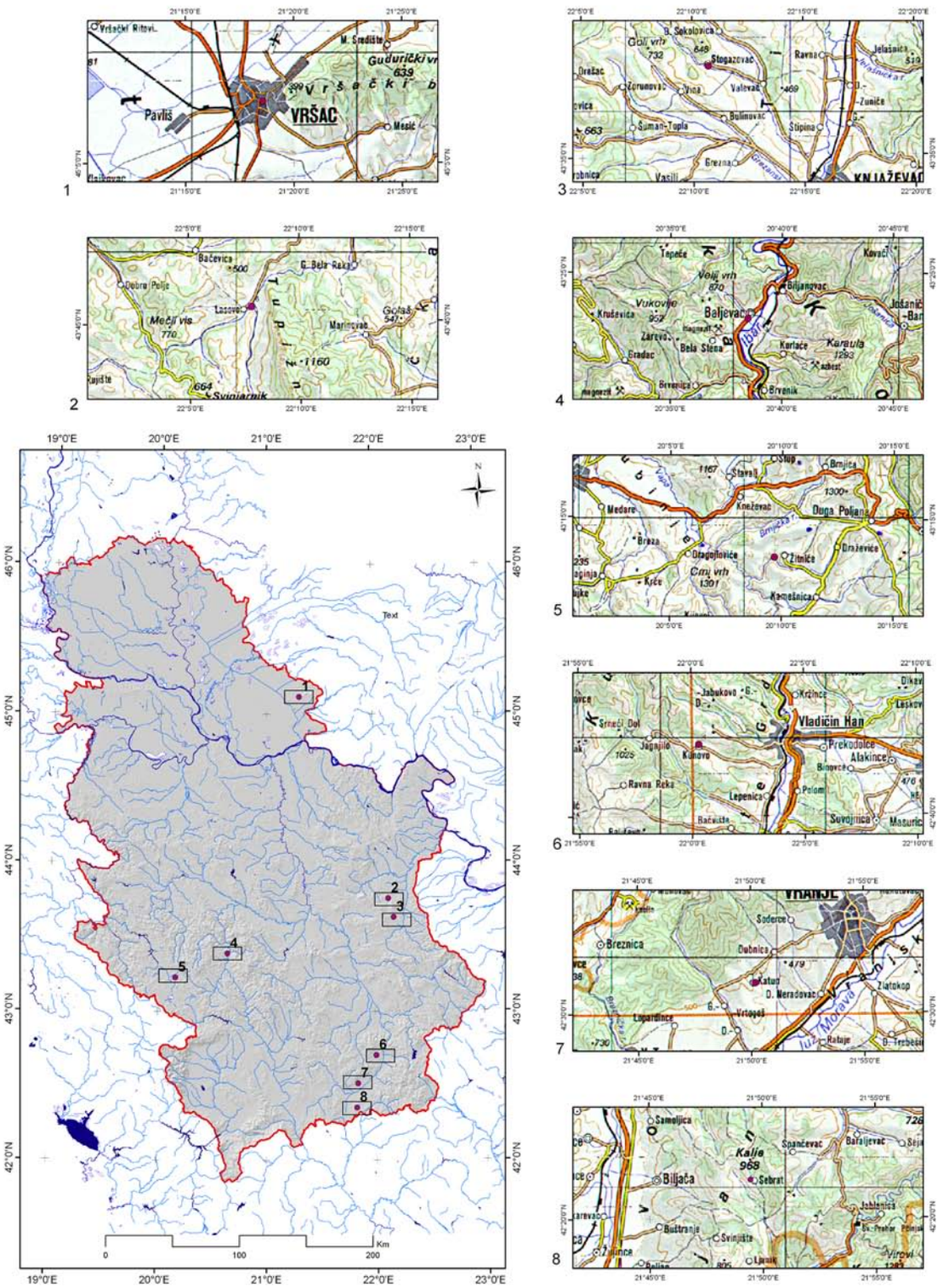

Fig. 1. Locations of sampling of *Apis mellifera carnica* in four geographical regions of Serbia.

The mtDNA region (starting at position 3363), including the $tRNA^{Leu}$ gene, the COI–COII intergenic region, and the 5' end of the COII subunit gene, was amplified by means of primers E2 (5′-GGCAGAATAAGTGCATTG-3′) and H2 (5′-CAATATCATTGATGACC-3′) and digested with DraI (Garnery et al., 1993). The PCR reaction mixture contained 1 x Taq buffer (Roche), 1.5 mM $MgCl_2$, 1μM of each primer, 1mM of each dNTP, 0.6 U of Taq polymerase (PE Applied Biosystems), and 1 μl of DNA extract. Conditions for the PCR reaction were as follows: initial denaturation (94°C, 3 min); 30 cycles of subsequent denaturation (94°C, 45 s); primer annealing (57°C, 45 s); and DNA extension (72°C, 1 min). All PCR amplifications were perfomed in a programmed thermocycler system, namely GeneAmp® PCR System 9700 (AB Applied Biosystems). Quantity (size) of the amplified COI-COII region was determined with a 7-μl aliquot of PCR product by electrophoresis on 1.5% agarose gel.

All reactions for sequencing were prepared using BigDye Terminator Ready Reaction Mic (PE Applied Biosystems) as per producer recommendations. The COI-COII region was sequenced by means of the E2 primer. The PCR reaction was stopped in the programmed thermocycler under the following conditions: 10 s denaturation at 96°C, 5 s of annealing at 50°C, and 4-min extension at 60°C, repeated for 30 cycles. Amplified, fluorescently assigned, and ruptured DNA was salted out and analyzed in an ABI Prism 310 automatic sequencer (Perkin Elmer).

Sequences of the 5'-end of mtDNA (490 bp) were aligned with published sequences of 49 different mtDNA honeybee haplotypes, along with 38 from the „C" phylogenetic group. Alignment of sequences was performed by the ClustalW method using MegAlign software (DNAStar, Lasergene MegAlign, 2006). Resulting in a consensus of the phylogenetic tree, the methods of maximal parsimony (MP) and neighbor-joining (NJ) analysis were performed using the same software. For estimates of the similarity index and evolutionary divergency between DNA sequences, Mega 4 softwer was used (Tamura et al., 2004, 2007).

RESULTS

It was discovered that the PCR-amplified COI-COII mtDNA region has a length of about 520 bp in all studied samples. To define the exact haplotypes, the PCR product of all samples was sequenced and the obtained sequences aligned with already published sequences of 49 different honeybee mtDNA haplotypes. Until only recently (Kozmus et al., 2007) it had been thought that all honeybee lines from Serbia studied so far belong to the C2D mtDNA haplotype of the C phylogenetic group. This C2D haplotype differs from the *A. m. carnica*-specific haplotype in two C-T transitions previously found only in samples of the *A. m. macedonica* and "Buckfast J" selected honeybee lines from Germany (Sušnik et al., 2004).

All our samples exhibited differences corresponding to the C2E haplotype described in Serbian honeybee samples as three polymorphic positions, viz., two A-T transversions and one insertion of nucleotide A (Kozmus et al., 2007). However, in bees of the Katun 7 group belonging to phylogenetic group C, one deletion of the T nucleotide at position 3534 was detected for the first time along with one G-A transition at position 3587 in phylogenetic group C2 in *A. m. carnica*. This transition was recently described in phylogenetic group C1 in haplotype C1F (Özdil et al., 2009). The numbers of mtDNA nucleotides are taken from Crozier and Crozier (1993). Since the sample from the Katun 7 group contained five individuals and since the same sequences were found in all samples, it can be concluded that group 7 represents a separate mtDNA haplotype special for only the small honeybee population from the vicinity of Vranje. We suggest that this new mtDNA haplotype in the subspecies *A. m. carnica* be assigned as C2I (Table 1).

Samples of the Stogazovac 3 group showed one T nucleotide deletion in position 3474 at a distance of 6 bp from the C nucleotide, already assigned as polymorphic position 2 (in the C2D haplotype). This matches a portion of the Timočki ecotype sample (T type) according to Kozmus et al. (2007). We suggest that this newly described mtDNA haplotype in the *A. m. carnica* subspecies be assigned as C2J

Table 1. Haplotypes and polymorphic nucleotides of the COI-COII intergenic region1 in the C2 phylogenetic line. (1) In this study, bold haplotypes were found, bold fragments and polymorphisms were novel, and shaded cells show polymorphisms found. The variable sites are marked as previously described in Franck et al. (2000a), where substitutions are labeled with numbers and insertion/deletion sites are labeled with lower case letters. The labels of the two new variable sites found in this study (h2, l2) are bold and in shaded cells.

MtDNA nucleotide positions (Crozier and Crozier, 1993)		3428	3442	3447	3467	3474	3486	3487	3493	3514	3534	3543	3567	3571	3573	3574	3575	3576	3577	3578	3587	3599	3601	3604	3632	3662	3691	3707	3761	3767	3769	3774	3816	3851
Haplotypes	GenBank Accession Numbers																																	
C1a (Franck et al., 2000)	FJ357798	C	T	-	C	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	T	A	T	T	T	A	T	C	C	C	T	A	T
C2a (Franck et al., 2000)	FJ357805	-	T	-	A	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C2b (Franck et al., 2000)	not submited	-	T	-	C	T	-	-	T	A	T	T	A	A	A	A	-	C	A	A	G	A	T	T	C	T	A	T	C	T	C	T	A	T
C2c (Sušnik et al., 2004)	not submited	-	T	-	C	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	A	T	T	T	T	A	T	C	T	C	T	A	T
C2d (Sušnik et al., 2004)	not submited	-	T	-	C	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	A	T	T	C	T	A	T	C	T	C	T	A	T
C2e (Kozmus et al., 2007)	not submited	-	T	-	C	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	A	T	T	C	T	A	T	C	T	C	T	A	T
C2f (Kandemir et al., 2006)	FJ357806	-	-	-	C	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C2g (Özdil et al., 2009)	FJ357807	-	-	-	C	T	-	-	T	T	T	T	T	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C2h (Özdil et al., 2009)	FJ357808	-	T	-	C	T	-	-	T	T	T	T	T	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	T	T	A	T
C2i (new)	FJ447491	-	T	-	C	T	-	-	T	T	-	T	A	A	A	A	-	C	A	A	A	T	A	T	C	T	A	T	C	T	C	T	A	T
C2j (new)	not submitted	-	T	-	C	-	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C11 (Solorzano et al., 2009)	FJ037776	-	T	-	C	T	-	-	T	T	T	T	T	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C12 (Solorzano et al., 2009)	FJ037777	-	T	-	C	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C14 (Solorzano et al., 2009)	FJ037778	-	T	-	C	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	T	A	T	C	C	A	T	C	T	C	T	A	T
C15 (Solorzano et al., 2009)	FJ037779	-	T	-	A	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C17 (Solorzano et al., 2009)	FJ037780	-	T	-	C	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	G	T
C18 (Solorzano et al., 2009)	FJ037781	-	T	-	C	T	-	-	T	T	T	T	A	A	T	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C19 (Solorzano et al., 2009)	FJ037782	-	T	-	C	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	A	T	A	T	C	T	A	T	C	T	C	T	A	T
C20 (Solorzano et al., 2009)	FJ037783	-	T	-	C	T	-	-	T	T	T	-	A	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C21 (Solorzano et al., 2009)	FJ037784	-	T	-	C	T	-	-	T	T	T	T	A	A	-	-	-	-	-	-	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C22 (Solorzano et al., 2009)	FJ037785	-	T	A	C	T	-	-	T	T	T	T	A	A	T	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C24 (Solorzano et al., 2009)	FJ037786	-	T	-	C	T	-	-	T	T	T	T	A	T	A	A	-	C	A	-	G	T	A	T	C	T	A	T	C	T	C	T	A	T
NN (Kandemir et al., 2006)	AY618912	-	-	-	C	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
(Kandemir et al., 2006)	AY618913	-	T	-	A	T	-	-	T	T	T	T	A	A	A	A	A	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
(Kandemir et al., 2006)	AY618914	-	T	-	C	T	A	-	T	T	T	T	T	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	T
C13 (Solorzano et al., 2009)	AY618915	-	T	-	C	T	-	-	T	A	T	T	A	A	A	A	-	C	A	A	G	T	A	T	C	T	A	T	C	T	C	T	A	C
(Kandemir et al., 2006)	AY618916	-	T	-	C	T	T	A	-	T	T	T	A	A	A	A	-	T	A	A	G	T	A	C	T	T	A	C	T	T	C	C	G	T
(Kandemir et al., 2006)	AY618917	-	T	-	C	T	T	A	-	T	T	T	A	A	A	A	-	T	A	A	G	T	A	C	T	T	A	C	T	T	C	C	G	T
(Kandemir et al., 2006)	AY618918	-	T	-	C	T	T	A	-	T	T	T	A	A	A	A	-	T	A	A	G	T	A	C	T	T	G	C	T	T	C	C	G	T
(Kandemir et al., 2006)	AY618921	-	T	-	C	T	-	-	T	T	T	T	A	A	A	A	-	C	A	A	A	T	A	T	C	T	A	T	C	T	C	T	A	T
Variable sites		*h*	h_1		*14*	**h_2**			*15*	**l2**		16_1					*17*			*19*				*21*	21_1			*24*	24_1				

(Table 1). The designations for the new haplotypes follow those of Franck et al. (2000a) and adhere to the order established to date for sequences from GenBank and published research papers.

The graph (Fig. 2) shows phylogenetic relations between sequences of the COI-COII mtDNA region in *A. m. carnica* from Serbia. The sequence of *A. m. ligustica* (Garnery et al., 1992) of the C1 phylogenetic group was used as an outgroup sequence.

Table 2 presents the results of estimating evolutionary divergence between sequences of the COI-COII mtDNA regions in the honeybee *A. m. carnica* from Serbia. A difference in the index of divergence between *A. m. carnica* from Serbia (belonging to the

Table 2. Estimates of evolutionary divergence between sequences of the COI-COII mtDNA intergenic region in honeybees (*A. m. carnica*) from Serbia. The number of base substitutions per site from analysis between sequences is shown. All results are based on the pairwise analysis of 10 sequences. Analyses were conducted using the maximum composite likelihood method in MEGA4. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 519 positions in the final dataset.

	A_m_ligustica	**Vršac_1**	**Lasovo_2**	**Stogazovac_3**	**Baljevac_4**	**Žitniće_5**	**Kunovo_6**	**Katun_7_ FJ447491**	**Sebrat_8**	**C2E**
A_m_ligustica	***									
Vršac_1	0.004	***								
Lasovo_2	0.004	0	***							
Stogazovac_3	0.004	0	0	***						
Baljevac_4	0.004	0	0	0	***					
Žitniće_5	0.004	0	0	0	0	***				
Kunovo_6	0.004	0	0	0	0	0	***			
Katun_7_ FJ447491	0.006	0.002	0.002	0.002	0.002	0.002	0.002	***		
Sebrat_8	0.004	0	0	0	0	0	0	0.002	***	
C2E	0.004	0	0	0	0	0	0	0.002	0	***

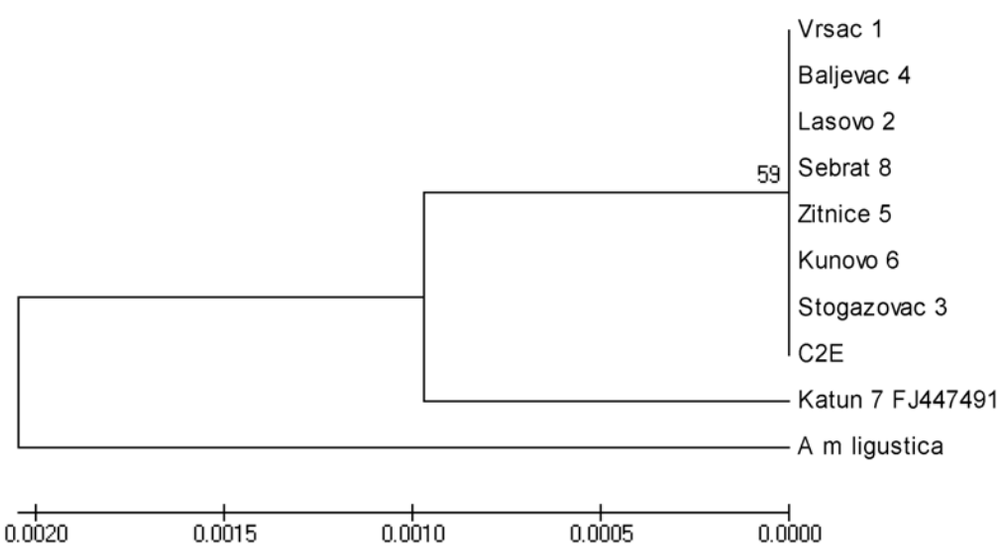

Fig. 2. Neighbor-joining (NJ) tree based on differences between COI-COII mtDNA region sequences in honeybees (*A. m. carnica*) from Serbia. The number above the tree branch indicates the percentage of the NJ tree supported by 1000 bootstrap replicates.

C2 phylogenetic group) and *A. m. ligustica* (belonging to the C1 phylogenetic group) can be clearly seen. The Katun 7 group is plainly dominant over all other lines in Serbia, which was confirmed with certainty by NJ phylogenetic analysis supported by 1000 bootstrap replicates (Fig. 2).

Comparative analysis of sequences of the COI-COII mtDNA region in the honeybee *A. m. carnica* from Serbia and other available sequences of the Mediterranean C2 phylogenetic group (with *A. m. ligustica* taken as an outgroup) from neighboring regions (mainly Turkey) showed consensus of MP and NJ (both methods revealed similar haplotype tree topology) in the phylogenetic tree (Fig. 3). Use of the similarity index to analyze mtDNA sequences in the *A. mellifera* C2 phylogenetic group shows a surprisingly high similarity of over 99.4% with

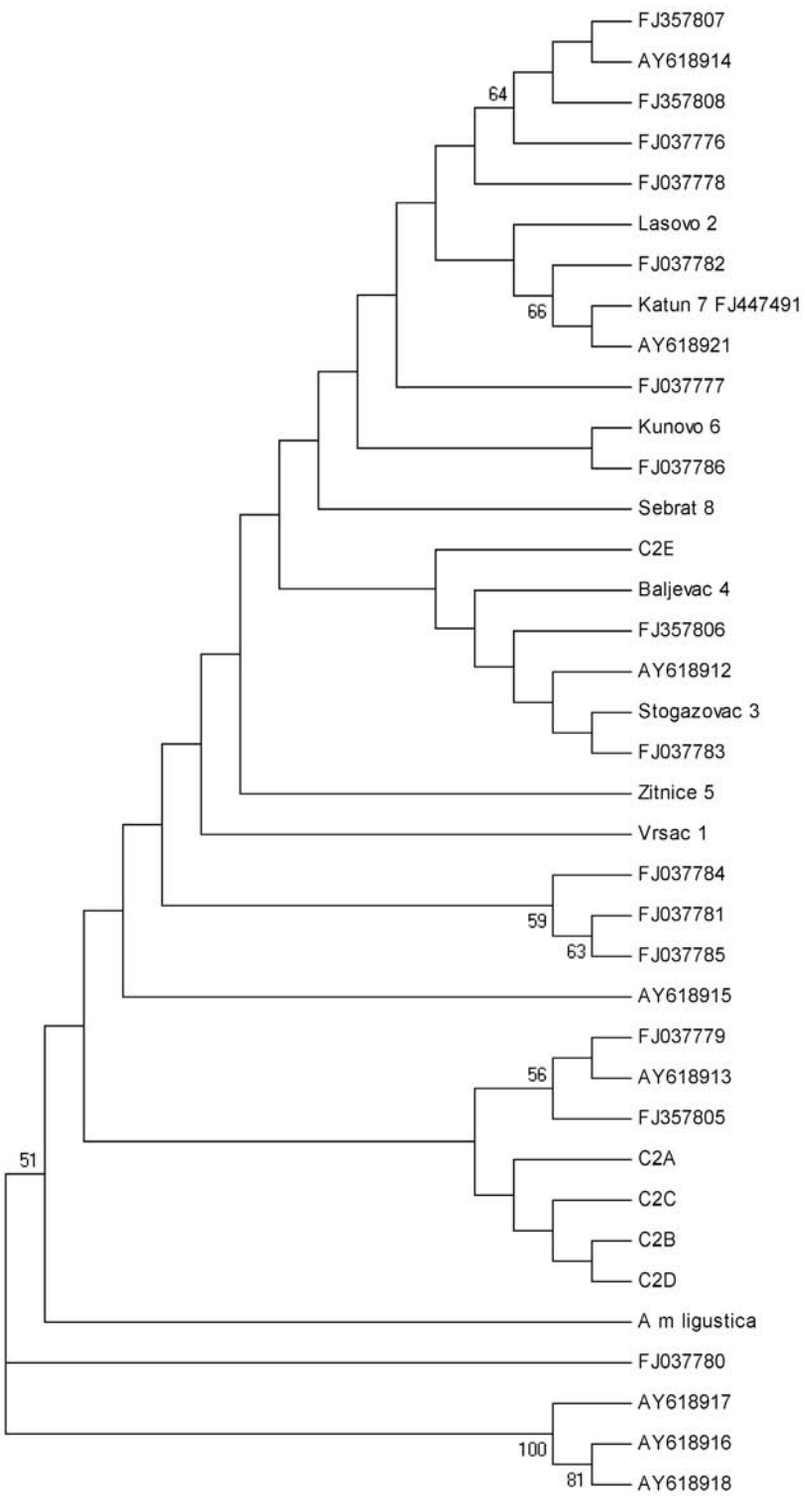

Fig. 3. Phylogenetic tree of the C2 phylogenetic line of *Apis mellifera* based on COI-COII region sequences as inferred by consensus of MP and NJ methods. The numbers above tree branches indicate the percentage of the tree topology supported by 1000 bootstrap replicates.

sequences of the same COI-COII mtDNA region in *A. m. cypria* from Turkey (GenBank access number AY618921).

DISCUSSION

Calculated distances and estimation of the evolutionary divergence between equalized (ClustalW in DNAStar, Lasergene MegAlign, 2006) COI-COII mtDNA sequences in the studied selected honeybee lines from Serbia distrinctly revealed prevalence of the Katun 7 line (Table 2). The difference in values of the similarity index and in the level of divergence relative to other lines is visually better perceived on the reconstructed phylogenetic tree obtained on the basis of the same molecular data (Fig. 2). In the phylogenetic tree (Fig. 2), it can be clearly seen that there exists a high degree of support (59%) on that part of the tree in which honeybees belonging to the Katun 7 group are dominant over all other Serbian honeybees. This can be one more reason for defining the Katun 7 group as a novel C2I mtDNA haplotype. The Stogazovac 3 group has one nucleotide T deletion at position 3474 (Table 1), which so far has not been recognized as a variable position in the COI-COII mtDNA region. We therefore suggest that a new designation (*h2*) be introduced for it as well. In accordance with the previously established way of describing mtDNA haplotypes, this deletion can also be considered as a new haplotype of *A. m. carnica* mtDNA with the new assignation of C2J. Phylogenetic analyses showed that this haplotype is very similar to all other haplotypes known in Serbia. Honeybees of the C2 phylogenetic group belonging to several different subspecies from the region of the Middle East (Turkey) display similarity with honeybees from Serbia (Fig. 3). On the basis of the conducted analysis, no separate grouping of individual honeybee subspecies according to mtDNA haplotypes was observed, except in the case of the three *A. m. syriaca* haplotypes. Analysis of sequences in the COI-COII mtDNA region in Serbian *A. m. carnica* lines and available reference data (BLAST browsing) shows a surprisingly high similarity (over 99.4%) between the newly described specific C2I mtDNA haplotype within the subspecies *A. m. carnica* and sequences of the same COI-COII mtDNA region in *A. m. cypria* from Turkey (GenBank access number AY618921) with high bootstrap support of 66% (Fig. 3). All the other studied lines of *A. m. carnica* from Serbia show a high similarity with this *A. m. cypria* haplotype. On the basis of so high a similarity, it could be supposed that in some former period there occurred hybridization between the domestic subspecies *A. m. carnica* and the Cyprian *A. m. cypria*. This assumption is supported by the fact that the Katun 7 honeybee line differs substantially in morphometric characteristics from other so far studied lines in Serbia (Nedić, 2009). A detailed taxonomic analysis (from the morphological to molecular level) needs to be carried out on honeybees from the vicinity of Vranje. Direct comparison with honey bees of the *A. m. cypria* subspecies will show whether their spreading has occurred, as suggested by the herein confirmed similarity between sequences in the COI-COII region of the mtDNA genome. It is known that the pattern typical of *A. m. carnica/A. m. ligustica* predominates throughout Turkey (97.9%) (Kandemir et al., 2006), which is confirmed by our analyses as well (Fig. 3), since the phylogenetic tree shows mingling of Serbian and Turkish haplotypes. According to Ruttner (1992), *A. m. cypria* belongs to the Oriental (O) group, while *A. m. carnica* belongs to the C group, but those differences at the subspecies level are not visible on the basis of sequences of the COI-COII region of mtDNA. Such morphological differences probably arise from the overall impact of the environment in the area of their dissemination.

The distinct separation of C2E, C2I, and C2J haplotypes in Serbia from other C mtDNA lines implies that honeybees from Serbia represent an indigenous gene pool within *A. m. carnica*, with a small number of specific populations created during evolution. Because the territory of the Balkan Peninsula was a refugium for many animal species during the period of the Ice Age in the Pleistocene (Schmitt, 2007), the honeybee haplotypes of Serbia could possibly be regarded as relict groups that survived in certain regions with specific climatic and biogeographic environmental conditions.

Phylogenetic studies on Mediterranean honeybees must also take into account the human

factor throughout history. The importation of foreign honeybee queen bees and frequent moving of beehives during the year represent major factors which influence modification of genetic structure in autochthonous populations by way of genetic introgression. For distinct separation of the areas of dissemination of certain honeybee subspecies, it is necessary to take into account both morphological markers and molecular markers using mtDNA and microsatellites.

This research revealed two novel mtDNA haplotypes (C2I and C2J), which so far have been discovered only in honeybees from Southern and Eastern Serbia. The presence of the C2I haplotype in honeybees from Southern Serbia and its genetic similarity with the *A. m. cypria* honeybee show unambiguously that in the past there occurred a modification of genetic structure in some populations due to the influence of environmental conditions. The possibility that some other still undiscovered honeybee mtDNA haplotypes will be found in this region is not unlikely, especially if we remember that the region has not been widely investigated.

Acknowledgments — We are very thankful to Dr. Peter Kozmus from Agricultural Institute of Slovenia, Ljubljana, Slovenia, who kindly helping during the lab work on mtDNA extraction, amplification and sequencing. The authors would like to express their gratitude to the Ministry of Science and Technological Development of the Republic of Serbia for financial support.

REFERENCES

Arias, M. C., and *W. S. Sheppard* (1996). Molecular phylogenetics of honey bee subspecies (*Apis mellifera* L.) inferred from mitochondrial DNA sequence. *Mol. Phylogenet. Evol.* **5,** 557-566.

Avise, J. C., Arnold, J., and *R. M. Ball* (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Ann. Rev. Ecol. Syst.* **18,** 489-522.

Bouga, M., Harizanis, P. C., Kilias, G., and *S. Alahiotis* (2005). Genetic divergence and phylogenetic relationships of honeybee *Apis mellifera* (Hymenoptera: Apidae) populations from Greece and Cyprus using PCR-RFLP analysis of three mtDNA segments. *Apidologie* **36,** 335-344.

Bowtell, D. D (1987). Rapid isolation of eukaryotic DNA. *Anal. Biochem.* **162,** 463-465.

Cornuet, J. M., Garnery, L., and *M. Solignac* (1991). Putative origin and function of the intergenic region COI and COII of *Apis mellifera* L. mitochondrial DNA. *Genetics* **1128,** 393-403.

Crozier, R. H., and *Y. C. Crozier* (1993). The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. *Genetics* **133,** 97-117.

De la Rúa, P., Gallián, J., Pedersen, B. V., and *J. Serrano* (2006). Molecular characterization and population structure of *Apis mellifera* from Madeira and the Azores. *Apidologie* **37,** 699-708.

De la Rúa, P., Gallián, J., Serrano, J., and *R. F. A. Moritz* (2001a). Genetic structure and distinctness of *Apis mellifera* L. populations from the Canary Islands. *Mol. Ecol.* **10,** 1733-1742.

De la Rúa, P., Gallián, J., Serrano, J., and *R. F. A. Moritz* (2001b). Molecular characterization and population structure of the honeybees from the Balearic islands (Spain). *Apidologie* **32,** 417-427.

DNAStar, Lasergene MegAlign (2006). *Software Suite for Sequence Analysis*, *ver. 7.1*. DNASTAR, Inc.

Franck, P., Garnery, L., Celebrano, G., Solignac, M., and *J. M. Cornuet* (2000b). Hybrid origins of honeybees from Italy (*Apis mellifera ligustica*) and Sicily (*A. m. sicula*). *Mol. Ecol.* **9,** 907-921.

Franck, P., Garnery, L., Loiseau, A., Oldroyd, B. P., Hepburn, H. R., Solignac, M., and *J. M. Cornuet* (2001). Genetic diversity of the honeybee in Africa: microsatellite and mitochondrial data. *Heredity* **86,** 420-430.

Franck, P., Garnery, L., Solignac, M., and *J. M. Cornuet* (1998). The origin of West European subspecies of honeybees (*Apis mellifera*): new insights from microsatellite and mitochondrial data. *Evolution* **52,** 1119-1134.

Franck, P., Garnery, L., Solignac, M., and *J. M. Cornuet* (2000a). Molecular confirmation of a fourth lineage in honeybees from the Middle East. *Apidologie* **31,** 167-180.

Garnery, L., Cornuet, J. M., and *M. Solignac* (1992). Evolutionary history of the honeybee *Apis mellifera* inferred from mitochondrial DNA analysis. *Mol. Ecol.* **1,** 145-154.

Garnery, L., Solignac, M., Celebrano, G., and *J. M. Cornuet* (1993). A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L. *Experientia* **49,** 1016-1021.

Kandemir, I., Kence, M., Sheppard, W. S., and *A. Kence* (2006a). Mitochondrial DNA variation in honeybee (*Apis mellifera* L.) populations from Turkey. *J. Apic. Res.* **45,** 33-38.

Kandemir, I., Meixner, M. D., Ozkan, A., and *W. S. Sheppard* (2006b). Genetic characterization of honeybee (*Apis mellifera cypria*) populations in Northern Cyprus. *Apidologie* **37,** 547-555.

Kozmus, P., Stevanović, J., Stanimirović, Z., Stojić, V., Kulišić, Z., and *V. Meglič* (2007). Analysis of mitochondrial DNA

in honeybees (*Apis mellifera*) from Serbia. *Acta Veter.* (*Belgrade*) **57 (5-6),** 465-476.

Meixner, M. D., Arias, M. C., and *W. S. Sheppard* (2000). Mitochondrial DNA polymorphism in honeybee subspecies from Kenya. *Apidologie* **31,** 181-190.

Meixner, M. D., Sheppard, W. S., and *J. Poklukar* (1993). Asymmetrical distribution of a mitochondrial DNA polymorphism between two introgressing honeybee subspecies. *Apidologie* **24,** 147-153.

Moritz, R. F. A., Cornuet, J. M., Kryger, P., Garnery, L., and *H. R. Hepburn* (1994). Mitochondrial-DNA variability in South African honeybees (*Apis mellifera* L.). *Apidologie* **25,** 169-178.

Nedić, N. (2009). *Biological-production characteristics of honeybees* (Apis mellifera carnica *Poll.*) *on the territory of Serbia*, 196 pp. Doctoral Thesis. Faculty of Agriculture, University of Belgrade, Belgrade.

Nedić, N., Mladenović, M., and *S. Rašić* (2007). Breeding program in beekeeping in Serbia, In: *Papers Presented at the 40th Apimondia International Apicultural Congress* (Melbourne, Australia), p.167.

Özdil, F., Yildiz, M. A., and *H. G. Hall* (2009). Molecular characterization of Turkish honeybee populations (*Apis mellifera* L.) inferred from mitochondrial DNA RFLP and sequence results. *Apidologie* (In press).

Palmer, M. R., Smith, D. R., and *O. Kaftanoglu* (2000). Turkish honeybees: genetic variation and evidence for a fourth lineage of *Apis mellifera* mtDNA. *J. Hered.* **91,** 42-46.

Ruttner, F. (1988). *Biogeography and Taxonomy of Honeybees*, 284 pp. Springer-Verlag, Berlin.

Ruttner, F. (1992). *Naturgeschichte der Hönigbienen*, 357 pp. Ehrenwirth Verlag, Munich.

Schmitt, T. (2007). Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. *Frontiers Zool.* **4,** 11-23.

Sheppard, W. S., Arias, M. C., Grech, A., and *M. D. Meixner* (1997). *Apis mellifera ruttneri*, a new honeybee subspecies from Malta. *Apidologie* **28,** 287-293.

Sheppard, W. S., Rinderer, T. E., Meixner, M. D., Yoo, H. R., Stelzer, J. A., Schiff, N. M., Kamel, S. M., and *R. Krell* (1996). *Hin*f1 variation in mitochondrial DNA of Old World honeybee subspecies. *J. Hered.* **87,** 35-40.

Smith, D. R., and *W. M. Brown* (1988). Polymorphisms in mitochondrial DNA of European and Africanized honeybees (*Apis mellifera*). *Experientia* **44,** 257-260.

Smith, D. R., Palopoli, M. F., Taylor, B. R., Garnery, L., Cornuet, J. M., Solignac, M., and *M. Brown* (1991). Geographical overlap of two mitochondrial genomes in Spanish honeybees (*Apis mellifera iberica*). *J. Hered.* **82,** 96-100.

Solorzano, C. D., Szalanski, A. L., Kence, M., McKern, J. A., Austin, J. W., and *A. Kence* (2009). Phylogeography and population genetics of honeybees (*Apis mellifera*) from Turkey based on COI-COII sequence data. *Sociobiology* **53 (2),** 237-246.

Stanimirović, Z., Stevanović, J., and *M. Andjelković* (2005). Chromosomal diversity in *Apis mellifera carnica* from Serbia. *Apidologie* **36,** 31-42.

Stevanović, J., (2002). *Investigation of morphometric and chromosomal variability in preserving the diversity of carniolan honeybee* (*Apis mellifera carnica Pollmann*, *1879*) *in Serbia*, 219 pp. MSc. Thesis. Faculty of Biology, University of Belgrade, Belgrade.

Sušnik, S., Kozmus, P., Poklukar, J., and *V. Meglič* (2004). Molecular characterization of indigenous *Apis mellifera carnica* in Slovenia. *Apidologie* **35,** 623-636.

Tamura, K., Dudley, J., Nei, M., and *S. Kumar* (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software, version 4.0. *Mol. Biol. Evol.* **24,** 1596-1599.

Tamura, K., Nei, M., and *S. Kumar* (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Nat. Acad. Sci. USA* **101,** 11030-11035.

МОЛЕКУЛАРНА КАРАКТЕРИЗАЦИЈА МЕДОНОСНЕ ПЧЕЛЕ *APIS MELLIFERA CARNICA* У СРБИЈИ

Н. НЕДИЋ[1], Љ. СТАНИСАВЉЕВИЋ[2], М. МЛАДЕНОВИЋ[1] и ЈЕЛЕНА СТАНИСАВЉЕВИЋ[2]

[1]*Пољопривредни факултет, Универзитет у Београду,* 11080 Београд, Србија
[2]*Биолошки факултет, Универзитет у Београду,* 11000 Београд, Србија

Анализиране су секвенце COI-COII региона митохондријалне ДНК код медоносних пчела из четири географски удаљена региона Србије (Вршац, Књажевац, Краљево и Врање). Истраживање је вршено на осам различитих, раније одабраних линија медоносне пчеле које се чувају-одржавају (линијска селекција) у оквиру четири репроцентра за матице. Све четири истраживане линије медоносне пчеле се на основу морфолошких и производних особина разликују, и специфичне су за одго-

варајући регион. Осим анализе секвенци мтДНК међу српским медоносним пчелама, извршена је и упоредна анализа са до сада познатим хаплотиповима C2 филогенетске линије. Резултати су открили два нова полиморфна места у COI-COII региону мтДНК, и то h2 на месту 3474 и l2 на месту 3534 (у оба случаја делеције T нуклеотида) међу медоносним пчелама у региону Врања и Књажевца, респективно. Описана су два нова мтДНК хаплотипа међу медоносним пчелама C2 филогенетске линије, и то C2I (ново поиморфно место l2 и G-A транзиција на месту 3587) и C2J (ново полиморфно место h2). Такође, упоредном анализом са секвенцама из ГенБанке података, утврђена је висока сличност (similarity index = 99.4 %) новог C2I мтДНК хаплотипа са једним хаплотипом *A. m. cypria* пореклом из Турске. Поједине популације домаће крањске медоносне пчеле из Србије могу претстављати аутохтони генски пул што може бити од великог значаја за даље очување биодиверзитета медоносне пчеле. Овим радом употпуњена је мтДНК карактеризација медоносне пчеле у Србији.