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The diagnostic utility of sequence-based assays for the molecular delimitation of the epidemiologically relevant *Culex pipiens pipiens* taxa (Diptera: Culicidae)

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Abstract

The northern house mosquito (*Culex pipiens pipiens* L.) is a vector of several important pathogens and comprises two epidemiologically distinct ecotypes (*molestus* Forskål and *pipiens*). The delimitation of its ecotypes is a crucial, yet controversial step in vector surveillance due to varying diagnostic values of different characters. Therefore, we reviewed the success of a diagnostic assay based on the mitochondrial cytochrome *c* oxidase subunit I locus (*COI*) by analyzing previously published sequences of *molestus* and *pipiens* sampled in different geographical areas. Next, by genotyping individuals from Northern Serbia at this locus, we additionally assessed whether genetic structure of urban and rural *Cx. p. pipiens* ecotypes corresponded to the admixture pattern. Finally, to account for the different susceptibility of genetic markers to introgression, we also analyzed genetic structuring based on the ribosomal internal transcribed spacer 2 (*ITS2*). No latitude-dependent differentiation of *Cx. p. pipiens* ecotypes was found at a global level, with the *COI* assay further failing to accurately identify *molestus* and *pipiens* ecotypes. Likewise, both individual- (BAPS) and population-based (analysis of molecular variance and F_{ST} estimates) methods showed no significant urban/rural genetic differentiation in Serbia, indicating unhindered gene flow between different *Cx. p. pipiens* habitat types. The findings challenge the previous instances of *Cx. p. pipiens* ecotype identification, while also spotlighting the vectorial capacity of their hybrid offspring.

Keywords: *COI* mtDNA, DNA barcoding, *ITS2* rDNA, *molestus*, *pipiens*

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Introduction

Accurate taxon identification is a crucial element of vector surveillance and control. Namely, different vector species exhibit varying potential to transmit pathogens, a property which depends on the vector's ecology and physiology (Talbalaghi & Shaikevich, 2011), and it is therefore important to precisely determine the vector composition of a certain area. This problem is particularly prominent in species complexes as they represent groups of taxa that are morphologically difficult or impossible to distinguish and which often showcase sharp differences in physiological and behavioral properties, the traits which influence their vectorial capacity and epidemiologic significance (Bahnck & Fonseca, 2006). To overcome this setback, a plethora of molecular approaches for taxon identification was developed, the most popular one being DNA barcoding (Hebert *et al.*, 2003).

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One of the vector species complexes whose taxonomy represents an outstanding problem is the *Culex* (*Cx.*) *pipiens* complex. It is a cosmopolitan mosquito assemblage that includes vectors of various viral, protozoan, and metazoan pathogens of people and animals. Some of the associated diseases include: West Nile encephalitis, Japanese encephalitis, avian malaria and filariasis (Shaikevich, 2017), and the spectra of affiliated pathogens keeps expanding with the emergence of novel studies (see El-Kholy *et al*., 2017 for hepatitis C virus implications). Despite physiological and behavioral differences, the members of the *Cx. pipiens* complex exhibit a high degree of morphological similarity, making it difficult to precisely discriminate between them (Becker *et al*., 2012). The morphological similarity of the taxa included in the assemblage is particularly pronounced in its nominal species, *Cx. pipiens* L. Namely, it includes two subspecies, *Cx. p. pipiens* and *Cx. p. pallens* Coquillett (www.mosquito-taxonomic-inventory.info), with the former further comprised of two morphologically almost identical, yet physiologically and behaviorally distinct ecotypes: *molestus* Forskål (autogenous, stenogamous, homodynamic, and mammophilic) and *pipiens* (anautogenous, eurygamous, heterodynamic, and ornithophilic) (Becker *et al*., 2012). Their physiological and behavioral differences are interpreted as adaptations to different habitats: while *molestus* mosquitoes usually inhabit underground, urban areas, *pipiens* mosquitoes are usually found in aboveground, rural habitats (Chevillon *et al*., 1995).

Due to their epidemiological distinctiveness (Di Luca *et al*., 2016), it is necessary to delineate the two *Cx. p. pipiens* ecotypes. However, apart from generally unfruitful attempts using morphological characters (see Kent *et al*., 2007 for summary, but also Vinogradova *et al*., 1996; Krtinić *et al*., 2012, 2015), their delimitation is problematic since they were defined using phenotypic differences, which are prone to environmental modulation (Chevillon *et al*., 1995). To overcome these difficulties, the issue of the *molestus/pipiens* differentiation has been tackled by a multitude of molecular approaches. Some of the markers include: the 3′ end of mitochondrial cytochrome *c* oxidase subunit I gene (Shaikevich *et al*., 2005; Kent *et al*., 2007), microsatellite loci (Fonseca *et al*., 2004; Kent *et al*., 2007; Gomes *et al*., 2009, 2013; Kothera *et al*., 2013), the flanking region of *CQ11* microsatellite locus (*CQ11FL*) (Bahnck & Fonseca, 2006; Gomes *et al*., 2009, 2013; Di Luca *et al*., 2016), intergenic spacer (Shaikevich *et al*., 2013), *12S* rRNA, *HS60*, *ND4* (Kent *et al*., 2007), and most recently, *period* and *timeless*, circadian rhythm genes (Shaikevich *et al*., 2016), as well as a genome-wide AFLP approach (Gomes *et al*., 2015). The issue of *molestus/pipiens* identification, however, remains a controversial topic since different DNA regions are subject to varying levels of gene flow as a consequence of their mode of inheritance (Petit & Excoffier, 2009), which is reflected in their diagnostic value.

Moreover, a regionally varying migration/selection balance was proposed in *Cx. p. pipiens* ecotypes (Chevillon *et al*., 1998). Namely, it has been suggested that the degree of genetic exchange between *molestus* and *pipiens* populations changes in a latitude-dependent manner, being the greatest in the Mediterranean region and gradually declining toward northern areas (Chevillon *et al*., 1998). Such a strengthening of the genetic connectedness of below- and aboveground *Cx. p. pipiens* populations was accompanied by a decreased selection against maladaptive alleles in aboveground populations. Hence, putatively diagnostic alleles may be variably distributed among geographic *molestus* and *pipiens* populations, due to the varying balance between homogenizing gene flow and divergent selection (see Chevillon *et al*., 1998 for *Aat-1* locus example).

The success of *Cx. p. pipiens* ecotype delimitation therefore depends not only on the choice of a genetic locus, but also on the geographic region where the mosquitoes were sampled due to the different degree of interecotypic hybridization. Therefore, the diagnostic value of molecular assays which were only locally tested should be evaluated on a larger spatial scale. The establishment of regional DNA barcode libraries of mosquito species drove the accumulation of mitochondrial and nuclear sequences of *molestus* and *pipiens* ecotypes which were not necessarily discussed in regards to their delimitation. Consequently, it became possible to transregionally review molecular assays proposed for *Cx. p. pipiens* ecotype designation. Therefore, the first goal of our study was to assess the applicability of assays based on the sequence polymorphism in the 5′ end of mitochondrial cytochrome *c* oxidase subunit I (5′ *COI* mtDNA) and internal transcribed spacer 2 (*ITS2* rDNA) loci, which were so far locally tested [e.g., in Russia (Shaikevich, 2007) and the UK (Danabalan *et al*., 2012) for 5′ *COI* mtDNA and the USA (Crabtree *et al*., 1995) and Russia (Vinogradova *et al*., 2007) for *ITS2* rDNA] and suggested as diagnostic and not diagnostic, respectively. Next, we investigated whether the patterns of genetic structuring on a global scale using these two markers would correspond to *molestus/pipiens* sample grouping. The use of both 5′ *COI* mtDNA and *ITS2* rDNA allowed us to study the pattern of genetic differentiation using markers which differed in inheritance mode (uniparental vs. biparental inheritance) and mutational rate (mitochondrial vs. nuclear gene). Finally, concerning *molestus/pipiens* designation, we wanted to identify *Cx. p. pipiens* mosquitoes sampled in Serbia using these two assays in the case of their applicability, and compare the results with *CQ11FL* identification, suggested as the most reliable locus for this purpose (Di Luca *et al*., 2016).

A separate issue important for vector and disease control is the scale at which management strategies should be implemented. Namely, in the case of gene flow between urban and rural areas, pathogens and insecticide resistance alleles could be exchanged between them. Therefore, it is also necessary to investigate the genetic connectedness of urban and rural mosquito samples (see Krtinić *et al*., 2014). Thus, we also genotyped *Cx. p. pipiens* individuals originating from aboveground urban and rural samples in the Vojvodina Province (Serbia) at *COI* mtDNA and *ITS2* rDNA loci to test whether gene flow kept them connected.

Materials and methods

The transregional review of 5′ COI *mtDNA and* ITS2 *rDNA assays*

To review the diagnostic value of 5′ *COI* mtDNA and *ITS2* rDNA assays for *Cx. p. pipiens* ecotype identification, we analyzed sequences previously published in GenBank (table 1). Out of the total number of 5′ *COI* mtDNA and *ITS2* rDNA *Cx. p. pipiens* sequences present in GenBank, we selected a subsample where the mosquitoes were identified up to the ecotype level using one or several ecotype-defining criteria (table 1). Additionally, sequences obtained *de novo* in this study from mosquitoes sampled on the territory of the Autonomous Province of Vojvodina were included (to determine with which ecotypes they cluster together), making the

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Table 1. Ecotype membership, region of origin, source article, ecotype-defining criteria, GenBank accession codes, sample size, and *COI* mtDNA haplotype/*ITS2* rDNA allele representation of *Cx. p. pipiens* mosquitoes whose DNA sequences were retrieved from the GenBank and analyzed in the study.

Ecotype	Origin	Source	Criteria	Accession code	N^1	*COI* mtDNA	*ITS2* rDNA
Molestus	The UK	Danabalan *et al.* (2012)	F	JQ253834-40	7	I	–
	Germany	Shaikevich (2017)	E	HM008667, HM008670-1	3	II	–
	Austria	Zittra *et al.* (2016)	F	KU756486	1	I	–
	Italy	Di Luca *et al.* (2016)	F	KP728861-71	11	III	–
	Russia	Shaikevich (2007)	A, B	AM403492	1	II	–
		Shaikevich & Zakharov (2010)	B	FN395171-80	10		
	Turkey	Gunay *et al.* (2015)	E	KJ012149-61	13	II	–
	South Korea	Cho *et al.* (2013)	C	KC407755-9	5	II	–
	Australia	Batovska *et al.* (2016)	C	KU495005-8	4	I	–
Pipiens	The UK	Danabalan *et al.* (2012)	F	JQ253841-47	7	I	–
	Germany	Shaikevich (2017)	E	HM008665-6, HM00868-9	4	I	–
	Austria	Zittra *et al.* (2016)	F	KU756485	1	I	–
	Italy	Di Luca *et al.* (2016)	F	KP728846-60	15	II	–
	Russia	Shaikevich (2007)	A, B	AM403476	1	I	–
		Shaikevich & Zakharov (2010)	B	FN395181-5, FN395187-9	8	I	
				FN395186, FN395190	2	V	
	Turkey	Gunay et al. (2015)	E	KJ012110-48	39	I	–
Hybrid	Austria	Zittra *et al.* (2016)	F	KU756487	1	I	–
Molestus	The USA	Crabtree *et al.* (1995)	A	U22115-6	2	–	*L*
	Russia	Vinogradova & Shaikevich (2007)	A, B, D	AJ850084	1	–	*J*
				AJ850085-6	2		*A*
	Iran		G	EF539853	1	–	*H*
	Taiwan	Wang *et al.* (2017)	C	KU497626-8	3	–	*B*
	Australia	Batovska *et al.* (2017)	C	KU495641	1	–	*A*
				KU495642	1		*O*
				KU495643	1		*K*
				KU495644	1		*B*
				KX866003	1		*C*
				KX866004	1		*N*
Pipiens	The USA	Crabtree *et al.* (1995)	A	U22111-2	2	–	*S*
				U22113	1		*I*
				U22114	1		*R*
				U22117-8	2		*P*
	Sweden	Crabtree *et al.* (1995)	A	U22119-20	2	–	*M*
	Iran		G	EF539854	1	–	*D*
				EF539855	1		*F*
				EF539856	1		*A*

[1]N – number of individuals per region.
Criteria: A – autogeny, B – habitat type, C – distributional data, D – siphonal index of larvae, E – *COI* mtDNA assay, F – *CQ11FL* assay, G – unknown.

total of analyzed sequences 159 (26 Serbian and 133 from GenBank) and 47 (21 Serbian and 26 from GenBank) for 5′ *COI* mtDNA and *ITS2* rDNA marker, respectively. In the case of 5′ *COI* mtDNA, the primer combination we used (discussed below) amplified a fragment which is larger than the standard, approximately 650 bp-long barcode fragment, amplified by the LCO-1490/HCO-2198 (Folmer *et al.*, 1994) primer pair. Furthermore, as different primer combinations were used to sequence 5′ *COI* mtDNA of *Cx. p. pipiens* (see Batovska *et al.*, 2016), after the alignment of GenBank accessions with our sequences, an overlapping 5′ *COI* mtDNA fragment of around 500 bp was used for the review of the assay's diagnostic power. For *ITS2* rDNA, we used the entire amplified region of around 325 bp as it overlapped with the GenBank sequences. The number of haplotypes and variable positions was determined in DAMBE6 (Xia, 2017), while haplotype and allele networks were constructed in Network 5.0.0.0. (Fluxus Technology Ltd, Clare, Suffolk, the UK) using the median joining approach. The diagnostic power of DNA barcode assays was inferred from the existence of a barcode gap. Namely, when the range of interspecific diversity (in our case, divergence between the ecotypes) is greater than intraspecific diversity (here, distance within the ecotypes), the barcode gap is established and the barcoding approach is deemed successful (Puillandre *et al.*, 2011). The existence of the barcode gap between *molestus* and *pipiens* sequences was investigated by retrieving *p* distances between them using MEGA7 software (Kumar *et al.*, 2016).

Serbian sample genotyping and the analyses of genetic differentiation

The larvae of *Cx. p. pipiens* were collected from seven areas of the Autonomous Province of Vojvodina (leg. and det. Krtinić, B.) during the period from 2009 to 2010. The sampling design and map with localities of the studied Serbian samples was provided in detail by Krtinić with coauthors (Table S1, 2016). Data on 28 individuals were used for 5′ *COI* mtDNA and *ITS2* rDNA analyses (table 2). The mosquitoes were divided into two groups, defined by the aboveground habitat

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Table 2. Population origin, sample membership, sample size, code, and *COI* mtDNA haplotype/*ITS2* rDNA allele representation of the Serbian *Culex p. pipiens* individuals genetically analyzed in the study.

Origin	Abbreviation	Sample	DNA specimen code	*COI* mtDNA	*ITS2* rDNA
Subotica	SU	SII	NS671	H4	–
			NS672	H4	*g*
		SIII	NS669	H1	*a*
			NS670	H1	*a*
Kikinda	KI	SII	NS647	H3	*e*
			NS674	H1	*g*
			NS675	H1	*d*
		SIII	NS648	H1	*a*
			NS673	H4	*a*
Novi Bečej	NB	SII	NS678	H4	*c*
			NS679	H1	*f*
		SIII	NS676	–	*b*
			NS677	H4	–
Vrbas	VR	SII	NS645	H4	*a*
			NS646	H1	–
			NS668	H1	*g*
Novi Sad	NS	SII	NS577	H1	–
			NS578	H1	*a*
			NS579	H1	*g*
		SIII	NS580	H1	*a*
			NS581	H1	*g*
			NS680	H2	*a*
			NS681	H1	*g*
Sremska Mitrovica	SM	SII	NS666	H1	–
			NS667	H1	*g*
		SIII	NS664	H1	–
Alibunar	AL	SII	NS643	H1	–
			NS644	–	*a*
		SIII	–	–	–

type where the larvae were sampled (*sensu* Krtinić *et al*., 2014): SII sample, draining ditches and ponds in the city (urban habitat), and SIII sample, ponds and swamps outside urban area (rural habitat). The belowground, urban sample (SI) from the previous studies (Krtinić *et al*., 2012, 2014, 2015) was not included since only the sample from Novi Sad exists and we were interested in the assays' behavior at the regional (Vojvodina Province) scale. Following the metamorphosis of larvae into pupae and adults, the collected mosquitoes were determined as *Cx. p. pipiens* based on morphological characters (Gutsevich *et al*., 1974; Božičić, 1985).

The maceration of tissue and the extraction of total DNA was carried out using NucleoSpin® Tissue DNA extraction kit (MACHEREY-NAGEL, Düren, Germany) following the manufacturer's protocol, after which the DNA was amplified by PCR using an illustra PuReTaq Ready-To-Go PCR Beads kit (GE Healthcare Life Sciences, Buckinghamshire, the UK). To amplify 5′ *COI* mtDNA, we used the forward LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′, Folmer *et al*., 1994) and reverse Inger (5′-AAAAATGTTGAGGGAAAAAT GTTA-3′, UEA8 in Lunt *et al*., 1996) primer pair, while the amplification of *ITS2* rDNA was done using the forward IT S2A (5′-TGTGAACTGCAGGACACAT-3′) and reverse ITS2B (5′-TATGCTTAAATTCAGGGGGT-3′) primer pair (Beebe & Saul, 1995), with PCR conditions as described in Milankov *et al*. (2009). To check the success of reactions, amplification products were separated on a 2% agarose gel. PCR products were then purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Vilnius, Lithuania), and bidirectionally sequenced on ABI3730XL by Macrogen (The Netherlands).

Chromatograms obtained by 5′ *COI* mtDNA and *ITS2* rDNA sequencing were checked in Chromas 2.6 (Tehnelysium Pty Ltd, South Brisbane, Australia) for erroneously called bases, while sequence alignment was performed in BioEdit 7.2.5 (Hall, 1999). The retrieved 5′ *COI* mtDNA and *ITS2* rDNA sequences were uploaded to GenBank (MK607060-085 and MK584719-739). The number of haplotypes and variable positions was determined in DAMBE6 (Xia, 2017), while haplotype and allele networks were constructed in Network 5.0.0.0. (Fluxus Technology Ltd, Clare, Suffolk, the UK) using the median joining approach. The existence of stop codons within the 5′ *COI* sequences was investigated by using an online Translate tool (https://web.expasy.org/translate/).

Mosquitoes belonging to the *Cx. p. pipiens* urban and rural samples from Serbia were also identified to the ecotype level using the *CQ11FL* assay (Bahnck & Fonseca, 2006). The ecotype-specific alleles were amplified using primers CQ11F2 (5′-GATCCTAGCAAGCGAGAAC-3′), pipCQ11 (5′-CATGTTGAGCTTCGGTGAA-3′), and molCQ11 (5′-CCC TCCAGTAAGGTATCAAC-3′), while PCR conditions were as defined by Bahnck & Fonseca (2006). The reaction products were separated on a 2% agarose gel and visualized under UV light. The band characteristic for the *pipiens* ecotype is around 200 bp-long and (around 70 bp) shorter than the one characteristic for *molestus*, while hybrids are characterized by the presence of both alleles (Bahnck & Fonseca, 2006).

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The analyses of genetic differentiation were performed transregionally (global *molestus* vs. *pipiens* ecotype sample) and intraregionally (Serbian urban vs. rural sample). We applied the Bayesian model-based clustering algorithm implemented in BAPS 6.0 (Corander & Tang, 2007), jointly assigning 5′ *COI* mtDNA and *ITS2* rDNA GenBank sequences to artificial populations corresponding to the regions of origin. Clustering among populations was performed using Clustering with linked loci and codon linkage model. We ran BAPS for values of *K* ranging from one to ten, performing five replicates for each *K*. The results of the population mixture analysis were used as an input file for population admixture analysis using Admixture based on mixture clustering method. The number of iterations was set to 100, the number of reference individuals per population to 200, and the number of iterations per reference individual to 20. The degree of genetic differentiation between the samples in Serbia was also determined based on 5′ *COI* mtDNA and *ITS2* rDNA in BAPS 6.0 using identical settings. Apart from this individual-based analysis, genetic structuring in Serbia was also investigated using population-based analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) performed in Arlequin 3.11 software (Excoffier *et al*., 2005) using both markers. The mosquitoes sampled at different localities were pooled together as members of SII (urban) and SIII (rural) samples. Ten thousand permutations were used to determine the significance of variance components. Genetic differentiation, measured as Wright's F_{ST} (Weir & Cockerham, 1984), was also estimated among samples (urban and rural ecotypes of each locality were considered separately) using Arlequin 3.11, and the significance between each comparison pair was evaluated through 1000 permutation procedures.

Results

Identification and delimitation of Culex p. pipiens *ecotypes at the transregional scale*

The transregional review of the assays' diagnostic utility encompassed 159 5′ *COI* mtDNA and 47 *ITS2* rDNA sequences. The sequences yielded five 5′ *COI* mtDNA haplotypes (HI–HV) and 22 *ITS2* rDNA alleles (*A*–*V*; fig. 1), with four and 45 variable positions in total, respectively. No stop codons were registered in the 5′ *COI* mtDNA sequences and HII–HV differed from HI in a single substitution each. Furthermore, using the *CQ11FL* assay, all the SII and SIII *Cx. p. pipiens* individuals sampled in Serbia were identified as *pipiens* ecotype (Supplementary Fig. S1).

The lineage sorting between *Cx. p. pipiens* ecotypes was incomplete since *molestus* and *pipiens* shared both 5′ *COI* mtDNA haplotypes (two haplotypes: HI and HII) and *ITS2* rDNA alleles (one allele: allele A). Furthermore, with the most common sequences (5′ *COI* mtDNA HI and *ITS2* rDNA allele *A*, 54/121 and 13/47 individuals, respectively) being shared between *molestus* and *pipiens* individuals (between-ecotype *p* distance = 0%), these markers cannot be used to distinguish the two ecotypes due to the lack of a barcode gap. Furthermore, HIV has not been previously associated with either *Cx. p. pipiens* ecotype outside Serbia. This problem is particularly pronounced in the *ITS2* rDNA data set since six out of seven Vojvodina alleles have not been described before. Therefore, the discovery of novel, regionally private sequences further limits the utility of both molecular assays. We considered the possibility that the assay's diagnostic power may have been obscured by the use of different ecotype-defining criteria. Namely, *molestus* and *pipiens* mosquitoes whose sequences were harvested from GenBank were defined using one or more of the following criteria: autogeny, habitat type, distributional data, siphonal index of larvae and 5′ *COI* mtDNA and *CQ11FL* molecular assays (table 1). This is particularly noteworthy in the case of the distributional data, where *Cx. p. pipiens* mosquitoes in South Korea, Taiwan, and Australia were 'by default' treated as *molestus* ecotype since it is assumed that only this ecotype was introduced to urban environments in these regions (Farajollahi *et al*., 2011). The two criteria which have been considered reliable are autogeny and *CQ11FL* identification. Yet, when only a subset of mosquitoes defined by those two criteria is considered, 5′ *COI* mtDNA is still not sufficiently diagnostic to delimit the ecotypes. Namely, HI was shared by *pipiens* mosquitoes of the UK, Austria, Serbia, and Russia, and *molestus* mosquitoes sampled in the UK and Austria. Similarly, HII was represented by both *molestus* mosquitoes of Russia and *pipiens* mosquitoes sampled in Italy. Finally, the utility of the assay could be compromised by the high morphological similarity of *Cx. p. pipiens* and *Cx. torrentium* (Shaikevich & Zakharov, 2010). Therefore, we compared a 5′ *COI* mtDNA sequence from GenBank (AM403477) with the haplotypes retrieved from our sample and found that the *Cx. torrentium* sequence differed in 13 substitutions from HI (the most common *Cx. p. pipiens* haplotype). Thus, it is unlikely that sample misidentification affected the results.

In the transregional BAPS analyses of genetic structuring, both 5′ *COI* mtDNA- and *ITS2* rDNA-based estimations retrieved five as the most probable number of clusters (fig. 2). In the case of 5′ *COI* mtDNA, the overall *pipiens* sample originating from different countries was predominantly represented by the members of cluster 1 (around 80% of individuals, Supplementary Table S1), similarly to Serbian SII and SIII *pipiens* samples (around 90% of individuals). When it came to the *molestus* sample, it was mostly represented by members of cluster 2 (around 60%), but also by the members of cluster 1 and cluster 3 (22 and 20%, respectively). Furthermore, *molestus* and *pipiens* sample groups were not always assigned to different clusters (fig. 2). Namely, the majority of samples (11/17), consisting of *molestus*, *pipiens*, and hybrid mosquitoes were placed in a single cluster, showing no 5′ *COI* mtDNA genetic structuring corresponding to *molestus*/*pipiens* division (fig. 2). Even in the case of locally separated groups corresponding to *Cx. p. pipiens* ecotypes, Italian *pipiens* ecotype was assigned to a cluster where the majority of samples belonged to the *molestus* ecotype (4/5 groups). In the cases of Italy, Turkey, Germany, and Russia, *molestus* and *pipiens* ecotypes were indeed placed in alternative 5′ *COI* mtDNA clusters. Conversely, both ecotypes sampled in the UK and Austria were placed in the same 5′ *COI* mtDNA cluster.

In terms of *ITS2* rDNA-based differentiation, no overall pattern of genetic structuring was apparent, with both *molestus* and *pipiens* ecotypes highly admixed by members of different clusters (fig. 2, Supplementary Table S1). The alleles retrieved in our study were distributed in four groups: (1) private for *molestus*, (2) private for *pipiens*, (3) private for Serbian *pipiens* urban and rural samples, and (4) shared among *molestus*, *pipiens*, and Serbian *pipiens* SII and SIII mosquitoes (the most common allele, *A*).

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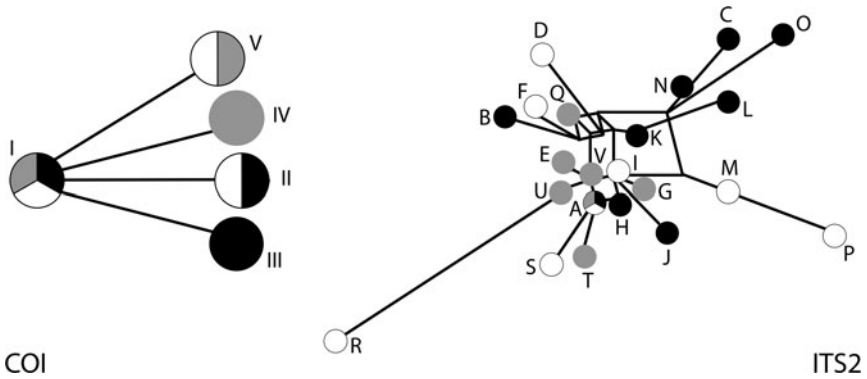

Fig. 1. Spanning network of *COI* mtDNA sequence haplotypes and *ITS2* rDNA alleles derived from the transregional *Cx. p. pipiens* ecotype analysis. Each circle represents one haplotype/allele, with black and white coloration indicating DNA sequences found in *molestus* and *pipiens* ecotypes, respectively, while grey coloration shows sequences found in the Vojvodina Province. The length of the branches connecting circles is proportional to the number of base differences between the respective sequences.

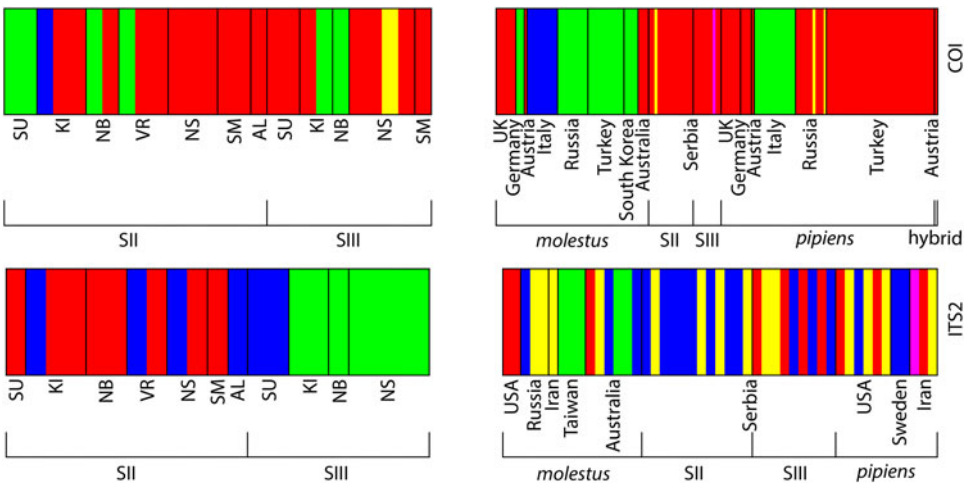

Fig. 2. Membership of the Vojvodina Province *Cx. p. pipiens* urban (SII) and rural (SIII) individuals (on the left) and *Cx. p. pipiens* ecotypes of the total sample (on the right), in a number of presumed clusters, as determined in BAPS using *COI* mtDNA and *ITS2* rDNA loci. An individual's probability of belonging to one of the clusters is represented by different colors.

Culex p. pipiens *aboveground urban and rural differentiation in Serbia*

In the intraregional analyses of genetic differentiation, 26 5′ *COI* mtDNA (around 900 bp-long) and 21 *ITS2* rDNA (around 325 bp-long) sequences were recovered from the Vojvodina Province since not all sequencing reactions were successful. The sequences yielded four 5′ *COI* mtDNA haplotypes (H1–H4) and seven *ITS2* rDNA alleles (*a*–*g*, fig. 3), with three and ten variable positions in total, respectively. Regarding 5′ *COI* mtDNA, H2, H3, and H4 differed from H1 in one substitution each, while *ITS2* rDNA alleles diverged up to four positions from allele *a*. No stop codons were registered in the 5′ *COI* mtDNA sequences.

Genetic differentiation between aboveground urban and rural *Cx. p. pipiens* samples on the territory of northern Serbia was assessed using 5′ *COI* mtDNA and *ITS2* rDNA by implementing individual- and population-based approaches. Genetic structure analyses performed in BAPS based on 5′ *COI* mtDNA and *ITS2* rDNA sequences retrieved four and three genetic clusters in Vojvodina, respectively (fig. 2). 5′ *COI* mtDNA genetic clusters corresponded to each haplotype registered in Vojvodina (H1–H4), while seven *ITS2* rDNA alleles were clustered in three distinct groups: alleles *a* and *b* were grouped together, allele *e* was singled out, and the remaining alleles *c*, *d*, *f*, and *g* were recognized as a separate group. In terms of the average membership coefficient of urban and rural *Cx. p. pipiens* samples, the 5′ *COI* mtDNA results showed no apparent distinction between mosquitoes of the SII and SIII samples as both groups were predominantly represented by the members of clusters 1 (around 70%) and 2 (around 20%) (Supplementary Table S1). However, in the case of *ITS2* rDNA, there was a notable degree of genetic differentiation, with SII mosquitoes predominantly represented by members of cluster 1 (67%) and SIII mosquitoes mainly represented by cluster 2 (78%). Furthermore, no cluster 1 members were registered in SIII sample, while no cluster 2 members were registered in SII sample. However, a portion of both

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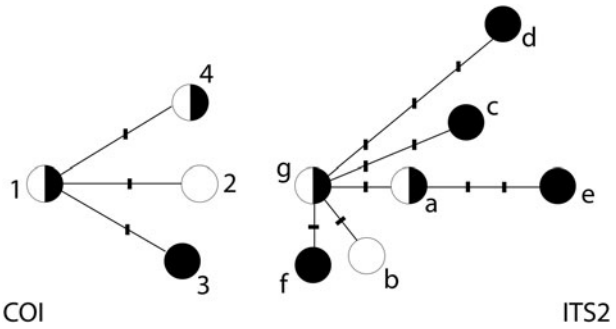

Fig. 3. Spanning network of mitochondrial cytochrome *c* oxidase subunit I DNA (*COI* mtDNA) sequence haplotypes and internal transcribed spacer 2 of ribosomal DNA (*ITS2* rDNA) alleles derived from the Vojvodina Province *Culex p. pipiens* populations. Each circle represents one haplotype/allele, with black and white coloration indicating DNA sequences found in urban (SII) and rural (SIII) samples, respectively. Each dash indicates a single base difference between the sequences.

samples' genetic structure was represented by members of cluster 3 (33% and 22% with respect to SII and SIII ecotypes).

Similarly, slightly discordant results of genetic differentiation were registered when population-based AMOVA analyses were implemented. The DNA barcode markers, 5′ *COI* mtDNA and *ITS2* rDNA, showed no genetic differentiation at varying hierarchical levels, both when the samples were divided into urban and rural groups and when they were considered together (ungrouped) (Supplementary Table S2).

Therefore, neither individual- nor population-based approaches of genetic differentiation showed concordance with the correlation between the habitat type where the mosquitoes were sampled in Vojvodina, except in the case of the individual-based approach using *ITS2* rDNA sequences.

Discussion

The diagnostic utility of 5′ COI *mtDNA and* ITS2 *rDNA assays in* molestus/pipiens *delimitation*

Sequence-based molecular approaches to *Cx. p. pipiens* ecotype designation predominantly used 5′ end of cytochrome *c* oxidase subunit I mtDNA (5′ *COI* mtDNA) as a DNA barcode marker (Shaikevich, 2007, 2017; Shaikevich & Zakharov, 2010; Danabalan *et al.*, 2012; Gunay *et al.*, 2015). This locus was firstly proposed by Shaikevich (2007) as diagnostic for *molestus*/*pipiens* delimitation when underground, autogenous populations in a European part of Russia were found to differ in one fixed substitution from aboveground, anautogenous populations (HII vs. HI labeled herein, respectively). A subsequent analysis of *Cx. p. pipiens* ecotypic differentiation on the same territory (Shaikevich & Zakharov, 2010) used the entire *COI* mtDNA sequence (around 1550 bp) to define three haplotypes (labeled A, B and C) associated with *pipiens* ecotype and a single, fixed haplotype (D) characteristic of *molestus* ecotype. When only 5′ *COI* mtDNA is considered, previously defined haplotypes A and C correspond to our HI, while B and D haplotypes correspond to our HV and HII. Since both HV and HII differ from HI in a single substitution, it is evident that there is no threshold value in 5′ *COI* mtDNA genetic distance which separates the two ecotypes.

DNA barcoding underwent conceptual developments since its inception as a distance-based molecular tool, assimilating the character-based framework of traditional taxonomy and recognizing taxon boundaries based on discrete nucleotide substitutions (character states) within a DNA sequence (DeSalle *et al.*, 2005). In this regard, 5′ *COI* mtDNA would still stand as a valid DNA barcode marker since a diagnostic transition of guanine to adenosine at the position 270 of 5′ *COI* mtDNA sequence used by Shaikevich & Zakharov (2010) distinguished *molestus* from *pipiens* mosquitoes. However, in our study, the transregional analysis of 5′ *COI* mtDNA barcode marker was not diagnostic for *molestus* and *pipiens* designation, with HI and HII being shared between them. Haplotype HI, which was proposed as specific for *pipiens* ecotype (Shaikevich, 2007), was registered in *molestus* mosquitoes in the UK (Danabalan *et al.*, 2012), Austria (Zittra *et al.*, 2016), and Australia (Batovska *et al.*, 2016), while HII was, apart from *molestus* mosquitoes, registered in *pipiens* mosquitoes in Italy (Di Luca *et al.*, 2016).

The practical outcome of this finding challenges studies which exclusively used the 5′ *COI* mtDNA assay to identify which *Cx. p. pipiens* ecotype was present in the studied region. Namely, Gunay *et al.* (2015) and Shaikevich (2017) used only the 5′ *COI* mtDNA assay to identify *Cx. p. pipiens* ecotypes, recognizing representatives of HI as *pipiens* and those of HII as *molestus*. However, their findings could easily be erroneous in the light of the presented results, and even epidemiologically irrelevant since the assay does not actually imply any strict correlation to physiological and behavioral traits. Furthermore, 5′ *COI* mtDNA was used for *molestus*/*pipiens* delimitation along with the *CQ11FL* assay. Although Danabalan *et al.* (2012) recognized 5′ *COI* mtDNA as a more robust molecular assay due to the unexpected presence of *molestus* mosquitoes in aboveground habitats in the UK, our results support Di Luca *et al.*'s (2016) conclusion that the *CQ11FL* assay is more accurate for *Cx. p. pipiens* ecotype identification.

Although *ITS2* rDNA proved successful for distinguishing the closely related species of the genus *Anopheles* (see Batovska *et al.*, 2017), we found it to be inefficient in *Cx. p. pipiens* ecotype designation. Similar to 5′ *COI* mtDNA, this marker cannot be used either as a genetic distance- or character state-based DNA barcode marker for identifying *molestus* and *pipiens* ecotypes. Whether *ITS2* rDNA can reliably distinguish between *Cx. p. pipiens* and the closely related *Cx. quinquefasciatus* remains questionable. Wang *et al.* (2017) successfully distinguished between the two taxa in Taiwan using this marker, but Batovska *et al.* (2017) failed to do so in Australia. Probably owing to its high mutational rate, these results also point out spatial *ITS2* rDNA variability: its utility as a DNA barcode marker to separate *Cx. p. pipiens* from *Cx. quinqefasciatus* varied between the continents and 20/22 alleles (table 1) in our study were private for the region where the mosquitoes were sampled.

In summary, neither 5′ *COI* mtDNA nor *ITS2* rDNA were useful in distinguishing *Cx. p. pipiens* ecotypes. Given that their success was not latitude-dependent, these assays should not be used for the identification of *molestus* and *pipiens* mosquitoes, especially since a better-suited marker exists (*CQ11FL*). A further advantage of this assay based on length polymorphism is its ability to distinguish *molestus* and *pipiens* hybrids from pure ecotype mosquitoes. However, a combination of several markers seems to give the best resolution for identifying *Cx. pipiens* complex members. Namely, *Cx. torrentium*, *Cx. quinquefasciatus*, and *Cx. pipiens* should be

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discriminated based on the polymorphism in intron 2 of the gene encoding acetylcholine esterase 2 (*ace-2*; Smith & Fonseca, 2004), while *CQ11FL* assay (Bahnck & Fonseca, 2006), despite the possibility of recombination obscuring the identification (Bahnck & Fonseca, 2006; Shaikevich & Vinogradova, 2014), should then be used for distinguishing between *molestus* and *pipiens* ecotypes.

The admixture of Cx. p. pipiens *urban/rural samples in Serbia and its epidemiologic consequences*

Concerning the differentiation between urban and rural samples of *Cx. p. pipiens* in Serbia, neither 5′ *COI* mtDNA nor *ITS2* rDNA indicated genetic differentiation between urban and rural *Cx. p. pipiens* samples in the population-based approach. Furthermore, we analyzed 306 individual genotypes from a previous study (Krtinić *et al*., 2016) using allozyme loci, performing both individual- (STRUCTURE) and population-based (AMOVA) analyses and obtaining the same urban/rural admixture pattern (data not shown). Such a concordance of markers which differ in evolutionary rate and inheritance mode suggests an intense gene flow between SII and SIII samples. Our results are compatible with the findings of Honnen & Monaghan (2018) who tested isolation by distance in *Cx. p. pipiens* mosquitoes of urban, peri-urban, and rural habitats in Germany using microsatellite loci, concluding that all the sampled individuals were derived from a single population with continuous gene flow through overlapping ranges. Furthermore, using the differences in electrophoretic mobility of hexokinase allozymes, Krtinić *et al*. (2014) previously recognized gene flow as a highly relevant microevolutionary process shaping genetic connectivity of urban and rural samples in Vojvodina Province. Therefore, although urban area exposes mosquitoes to the effects of air, water, and noise pollution (Honnen & Monaghan, 2018), gene flow still appears to keep the urban and rural habitats of *Cx. p. pipiens* genetically connected, as evidenced by both sequence-based (5′ *COI* mtDNA and *ITS2* rDNA) and multilocus markers (allozymes, microsatellite loci). The future studies should investigate whether intense gene flow characteristic of the supposedly neutral markers overpowers divergent selection acting upon the loci involved in specific adaptations to urban and rural habitats.

A well-recognized issue of *Cx. p. pipiens* mosquitoes is the hybridization of the two ecotypes since it can lead to an alteration of epidemiologically relevant traits (Fonseca *et al*., 2004). Namely, the hybrid offspring of ornithophilic *pipiens* ecotype and mamophylic *molestus* ecotype could act as the bridge vector promoting an exchange of avian and mammalian pathogens. In Serbia, such a scenario is supported by the registered outbreaks of West Nile virus (Popović *et al*., 2013; Kemenesi *et al*., 2014), clearly demonstrating the ability of *Cx. p. pipiens* mosquitoes to transfer an avian pathogen to human hosts. Furthermore, hybrids were detected in lower latitudes: Portugal (Gomes *et al*., 2009, 2013), Greece (Papa *et al*., 2013), and Italy (Di Luca *et al*., 2016), but also in higher latitudes: Austria (Zittra *et al*., 2016), the Netherlands (Reusken *et al*., 2010), and Germany (Rudolf *et al*., 2013), therefore supporting that gene flow between *molestus* and *pipiens* mosquitoes is not as restricted by latitude-dependent effect as thought previously. Finally, with gene flow keeping urban and rural samples connected, these effects of hybridization are not necessarily restricted to urban areas. Taking into the account and the possible spread of resistance alleles (between different urban areas, across rural areas between them), it is evident that studying microevolutionary processes which shape *Cx. p. pipiens* genetic structure remains an issue of high relevance for public health.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485319000105.

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