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CULEX TORRENTIUM (MARTINI), A NEW SPECIES IN CROATIAN MOSQUITO FAUNA

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In research into mosquitoes in the mountain regions of Croatia, a new species of mosquito for Croatian mosquito fauna has been identified. The *Culex pipiens* complex is represented with four sibling species. *Culex torrentium* belongs to this complex and morphologically differs from the other species only in the structure of male genitals. The presence of this species is determined based on the male genital structure and confirmed by mitochondrial cytochrome c oxidase molecular analysis. Specimens of these species have been found in all three mountain areas covered by this research: Slavonian mountains, Gorski Kotar area and central Velebit Mt. The lowest altitude where we found this species was 159 metres above sea level (m a.s.l.) in Pleternica, and the highest 906 m a.s.l. in Baške Oštarije. At all sites, the species *Cx. torrentium* was sampled in community with other species, but most frequently together with sibling species of *Cx. pipiens* (most probably the subspecies *Cx. p. pipiens*).

Key words: *Culex torrentium*, *Culex pipiens* complex, Croatia

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Faunističkim istraživanjem komaraca planinskih područja u Hrvatskoj zabilježena je nova vrsta komaraca za faunu Hrvatske. *Culex pipiens* kompleks predstavlja kompleks od četiri sestrinske vrste. *Culex torrentium* pripada tom kompleksu, a od drugih se vrsta razlikuje samo u građi genitalija mužjaka. Prisutnost ove vrste komaraca utvrđena je na osnovi razlika genitalija mužjaka, a potvrđena je molekularnom analizom mitohondrijalne citokrom c oksidaze. Jedinke ove vrste zabilježene su u svim istraživanim područjima u okviru ovog istraživanja: planine u Slavoniji, Gorskom Kotaru i srednjem Velebitu. Najniža nadmorska visina na kojoj je zabilježeno prisustvo ove vrste bila je 159 m u Pleternici, a najviša na 906 m u Baškim Oštarijama. Na svim postajama *Cx. torrentium* je zabilježen u zajednici s drugim komarcima, ali najčešće sa sestrinskom vrstom *Cx. pipiens* (najvjerojatnije podvrstom *Cx. p. pipiens*).

Ključne riječi: *Culex torrentium*, *Culex pipiens* kompleks, Hrvatska

INTRODUCTION

Various authors have described the *Culex pipiens* complex as several species, subspecies, forms, races, physiological variants, or biotypes. This complex includes the names *Cx. pipiens pipiens* Linnaeus, *Cx. p. pipiens* biotype *molestus* Forskal, *Cx. p. quinquefascia-*

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tus Say, *Cx. p. pallens* Coquillett, *Cx. restuans* Theobald, and *Cx. torrentium* Martini in the Holarctic as well as two Australian members, *Cx. australicus* Dobrotworsky and Drummond and *Cx. globocoxitus* Dobrotworsky (Becker *et al.*, 2010).

Cx. torrentium is considered to be a sibling species of the *Cx. pipiens complex*. Females and larvae of *Cx. pipiens* and *Cx. torrentium* are morphologically nearly identical and although for a long time, considered to occupy different habitats and geographical areas, they are well defined and separated species genetically. Based on larval collections and molecular species identification, recent studies have shown that the species often occur together, and that *Cx. torrentium* dominates in northern Europe and is as common as *Cx. pipiens* in Central Europe (Hesson *et al.*, 2015). Consequently, it is rare in Southern Europe. They share comparable ecological characteristics regarding the habitat of the adults as well as breeding sites and an almost identical morphology (Sherpner, 1960). The only reliable distinguishable morphological characteristic is the structure of the male hypopygium. *Cx. torrentium* has the dorsal arm of the aedeagus twisted and pointed apically and the ventral arm of paraproct well developed, strongly sclerotized. In contrast to *Cx. pipiens*, *Cx. torrentium* is adapted to lower water temperatures and dominates in cold regions or at higher altitudes (Schaffner *et al.*, 2001). Differences between sibling species *Cx. torrentium* and *Cx. pipiens* can be determined using mitochondrial cytochrome oxidase c subunit I. The most efficient approach in the differentiation of these two species is using both the morphological and the molecular approach. *Cx. torrentium* is present in many countries of Central Europe (Weitzel *et al.*, 2011), but its presence in Croatia was not confirmed until now.

The aim of this research was to investigate the presence of sibling species of *Cx. pipiens* and *Cx. torrentium* in Croatia. If it was present, our next aim was to determine how its range depends on altitude and climate conditions.

Study area

Sample collection was carried out in three areas (Fig. 1) of higher altitude for which there is little or no information on mosquito species. We chose the areas of Slavonian mountains, Gorski Kotar and the Central Velebit. All the areas are characterized by higher altitudes, more forest vegetation and higher rainfall than in the rest of Croatia, which contributes to the emergence of forest streams, lakes and ponds. Because of different soil permeability and dry weather during research we also paid attention to artificial water collections (e.g. tires, bath tubs, barrels).

The first area is located on the Slavonian mountains which surround the Požega valley. The selected transect included Mt Papuk and Požeška Gora with a maximum altitude of 515 m (forest near Novo Zvečevo) and minimum altitude of 177 m (forest near Požeška Koprivnica). The second area is located in Gorski Kotar from the foot of Samarske stijene (maximum altitude of 1011 m) to Brod na Kupi (minimum altitude of 234) in the north and Bribir in the south. Gorski Kotar is a plateau with an average height of 700-900 m from which the mountains range up to 1500 m. The climate is extremely montane. The third area is located in Central Velebit from Gospić in the east, Baške Oštarije and Brušane in the south and Krasno (Northern Velebit) in the north. The highest altitude was on the rocks behind Dabarski kukovi (1003 m) and in Štirovača (1012 m). The lowest altitude was in the village Kuterevo (529 m). Mt. Velebit is a natural border between continental and Mediterranean Croatia.

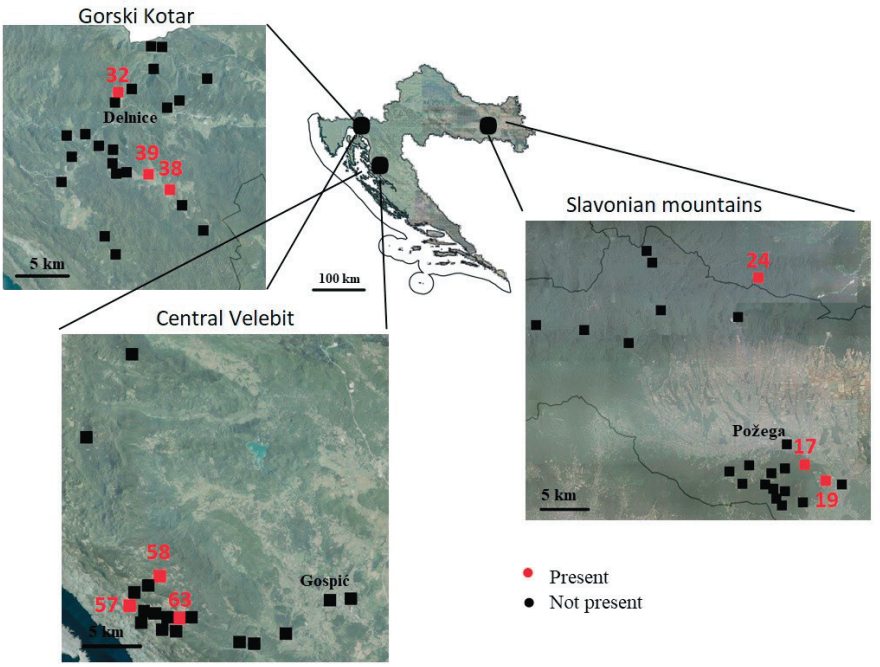

Fig. 1. Study area and locations where *Cx. torrentium* was recorded.

MATERIALS AND METHODS

The study was conducted during July and August 2017. Adult stages, as well as larvae were sampled at 68 localities, once per locality: 24 on the Slavonian mountains, 24 in Gorski Kotar and 20 in Central Velebit. Longitude and latitude of each locality were recorded using a geographical positioning system (GPS), and were later displayed using GoogleEarth.

Adult mosquitoes were caught using CDC traps in 30 of the 68 localities. Traps were baited using dry ice as attractant for mimicking CO_2 emission from live hosts. Adult mosquitoes were also collected using BG Sentinel traps with their usual attractant coupled with dry ice. BG Sentinel traps were placed aat 6 localities. All the traps were set in an appropriate habitat and were operated for at least 12 hours, including dusk and dawn. Sampled mosquitoes were stored in the entomological collection of the Department of Biology, Josip Juraj Strossmayer University of Osijek. Later, specimens were identified using the following keys: Becker *et al*. (2010), and Gutsevich *et al*. (1974).

Larvae were caught individually using a plastic dropper or using a net 25 cm in diameter with a dipper to collect the water containing the larvae. Larvae were collected from all natural and artificial habitats: ponds and channels and barrels and tyres, for example, respectively. Half of the collected specimens were brought to the laboratory and left to emerge so that we could obtain adult mosquitoes. After imagoes emerged they were either killed using potassium cyanide (KCN) and mounted on entomological pins following the identification using identification keys, or stored in 100% ethanol for later molecular identification to identify *Cx. torrentium*. The specimens

in the other were killed in the field by being placed in 100% ethanol for the making of permanent preparations. In some emerged male mosquitoes, (*Culex pipiens* complex) the genitalia were removed, exposed in hot KOH for 10 minutes, placed on a microscope slide in a drop of Canada balsam and covered with a coverslip. Afterwards, they were covered with a cover plate and identified using the identification keys Becker *et al*. (2010), and Gutsevich *et al*. (1974).

DNA was extracted from whole mosquito samples using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA fragment for mitochondrial cytochrome c oxidase subunit I (COI) was amplified using standard PCR protocol and cycling conditions (3 min at 94°C, 39 × (20 s at 94°C, 40 s at 52°C, 30 s at 72°C), 5 min at 72°C). PCR products were visualized using gel electrophoresis. PCR products were later sent for sequencing (Macrogen, Amsterdam, Netherland).

RESULTS

Specimens of the *Cx. pipiens* complex were sampled as adults and larvae. As adult mosquitoes cannot be determined to species level these samples were not taken into consideration in this paper (adult mosquitoes were caught in CDC as well as BG Sentinel traps). In total we found *Cx. pipiens* complex larvae in 26 sites. After emergence and analysis of male genital morphology, we noted the presence of *Cx. torrentium* at 9 sites. A total of 38 mosquitoes from these 9 sites were subjected to molecular analysis.

Presence of the *Cx. torrentium* species was determined using morphological analysis of male hypopygia and confirmed using molecular analyses. In three areas covered by this study we investigated 68 localities and in 9 we noted the presence of *Cx. torrentium* (Fig. 1). The presence was noted at 159, 183, 347 m a.s.l., in the Slavonian mountains, 712, 776 and 777 m a.s.l. in Gorski Kotar and 619, 712 and 906 m a.s.l. on Mt Velebit (Fig. 2).

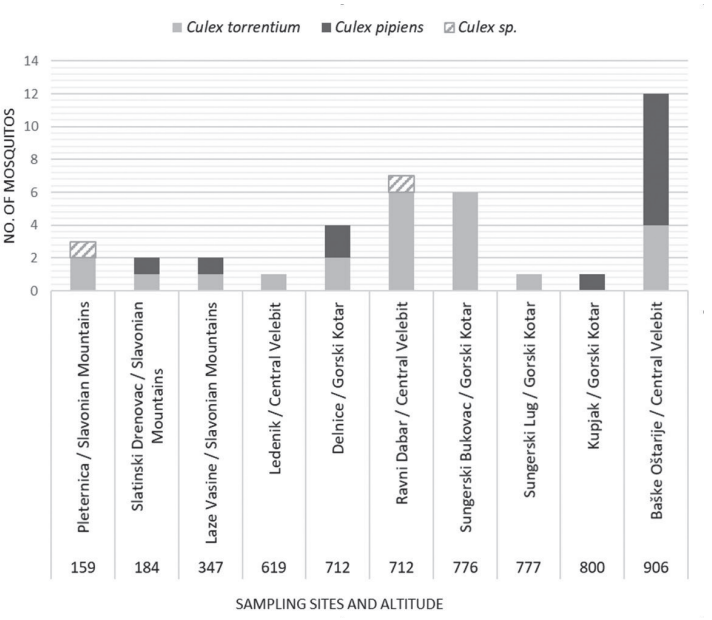

Fig. 2. Species composition of males from *Cx. pipiens* complex in investigated sites in the mountains of Croatia.

Analyzing the males from the *Cx. pipiens* complex we identified 38 individuals, of which 25 were identified as *Cx. torrentium*, 13 as *Cx. pipiens* and two remained undefined to species level and we noted them as *Culex* sp. Two sibling species, *Cx. pipiens* and *Cx. torrentium* occurred together at 4 sites of which two were at lower altitudes (<350 m a.s.l.) and two at higher altitudes (>600 m a.s.l.). *Cx. torrentium* was found alone in three sampling sites at higher altitudes (>600 m a.s.l.) (Fig. 2).

In almost all sites we noted the presence of *Cx. torrentium* with other mosquito species on the same sampling sites (Tab. 1). In the first area (Slavonian mountains), *Cx. torrentium* occurred together with *Cx. pipiens*, *Anopheles maculipennis* and *Aedes japonicus*, and in the second (Gorski Kotar) with *Cx. pipiens*, *Culex* sp., *Ae. japonicus*, *Culex hortensis*, *Culiseta longiareolata* and *Ochlerotatus* sp. In the third area (Central Velebit) *Cx. torrentuim* occurred with *Cx. pipiens*, *Culex* sp., *Ae. japonicus*, *Cx. hortensis*, *An. maculipennis*, *Cs. longiareolata*, *Culiseta annulata* and *Culiseta* sp.; in the Ledenik site we sampled *Cx. torrentium* only.

Tab. 1. Presence of *Culex torrentium* with other mosquito species in the larval stage on the same sampling sites.

Mark on the map	Location	Altitude	Habitat	Species
19	Pleternica / Slavonian mountains	159	Canal	*Culex pipiens*
				Culex territans
				Culex torrentium
				Culex sp.
				Anopheles maculipennis
24	Slatinski Drenovac / Slavonian mountains	184	Barrel	*Culex pipiens*
				Aedes japonicus
				Culex torrentium
				Anopheles maculipennis
17	Laze Vasine / Slavonian mountains	347	Well	*Culex pipiens*
				Culex torrentium
				Aedes japonicus
58	Ledenik / Central Velebit Mt.	619	Cemetery	*Culex torrentium*
32	Delnice / Gorski Kotar	712	Yard	*Culex pipiens*
				Culex torrentium
				Culex hortensis
				Culex sp.
				Aedes japonicus
57	Ravni Dabar / Central Velebit Mt.	712	Barrel	*Culex pipiens*
				Culex hortensis
				Culex sp.
				Culex torrentium
				Culiseta longiareolata
				Culiseta sp.
39	Sungerski Bukovac / Gorski Kotar	776	Barrel	*Culex pipiens*
				Culex torrentium
				Culex sp.
				Aedes japonicus
				Culiseta longiareolata
				Ochlerotatus sp.

Mark on the map	Location	Altitude	Habitat	Species
38	Sungerski Lug / Gorski Kotar	777	Canal	*Culex pipiens*
				Culex torrentium
63	Baške Oštarije / Central Velebit Mt.	906	Trough	*Culex pipiens*
				Culex torrentium
				Culiseta longiareolata
				Culiseta annulata
				Aedes japonicus
				Anopheles maculipennis

DISCUSSION

The species that we most often found to be dominant in this research belongs in the *Cx. pipiens* complex. Since it belongs among cosmopolitan species and was found up to 2500 m a.s.l. (Gutsevich *et al.*, 1974), this relatively low area is not a problem for their life. This species can be found everywhere, because it can breed in almost any kind of water habitat with any kind of food (Merdić, 1993). During our research, mosquitoes had large quantities of food available, so it is not unusual that we should have found them in large numbers.

These two species occur together in large areas of Europe; *Cx. torrentium* dominates in Northern Europe and *Cx. pipiens* dominates south of the Alps. The transition in dominance occurs in Central Europe, where both species are roughly equally common (Hensson *et al.*, 2014). There is a strong correlation between the length of the growing season at different sites and occurrences of the two species. As the growing season increases, the proportion and detection of *Cx. torrentium* decrease, whereas those of *Cx. pipiens* increase. In Croatia, the lower altitudes are very convenient for *Cx. pipiens*, but higher altitudes are similar to Northern Europe and better for the species which dominates in Northern Europe. It would be interesting for future research to find these mosquitoes at altitudes more than 1500 m a.s.l..

The presence of *Cx. torrentium* was confirmed using morphological and molecular analysis. *Cx. torrentium* populations were found in all areas covered by this research (Slavonian mountains, Gorski Kotar and Central Velebit). Distribution of *Cx. torrentium* depends on climate factors and the site altitude. In the area of the Slavonian mountains and Gorski Kotar, the distribution of *Cx. torrentium* probably depends more on the continental climate than on the higher altitudes. For example, in the area of the Slavonian mountains we found *Cx. torrentium* in lower altitudes on northern slopes and in fresh spring water, whereas at higher altitudes in warmer standing water we found both species. In the Gorski Kotar area we mostly found *Cx. torrentium*, probably due to the impact of the colder climate. In the area of central Velebit, where the impact of Mediterranean climate is stronger, both species are present at higher altitudes, but we found the dominance of *Cx. torentium* in north Velebit, which might depend on higher altitude only.

Different species of the *Cx. pipiens* complex are vectors for a number of arboviruses such as Japanese encephalitis, Sindbis and Usutu virus, but the West Nile Virus (WNV) is the most important for Europe (Becker *et al.*, 2010). The occurrence of WNV human infections in Croatia was recorded several years ago. The first outbreak of WNV neuroinvasive disease with seven human cases occurred in 2012 in three north-eastern

Croatian counties (Merdić *et al.*, 2012), after that, an outbreak occurred in Central and Northern Croatia (Vilibić Čavlek *at al.*, 2014). Human cases of WNV fever and WNV neuroinvasive disease were recorded in many Central European countries. During the 2017 transmission season, 288 human cases were reported in the EU and neighbouring countries. In addition, EU Member States reported 127 equine cases (ECDC, 2018). WNV is maintained in nature within an enzootic cycle involving ornithophilic mosquitoes and birds, but it can infect humans and equines (incidental or dead-end hosts). The *Cx. pipiens* complex members *Cx. pipiens* biotypes *pipiens* and *molestus,* as well as *Cx. torrentium,* are abundant in Central Europe (Hensson *et al.*, 2014). Both species preferentially take blood meals from birds, rendering them potential enzootic vectors for WNV in Central Europe. Slightly higher infection and dissemination rates even at a low average ambient temperature of 18°C have been proven in *Cx. torrentium* (Leggewie *et al.*, 2016).

The first record of *Cx. torrentium* in Croatia represents a new member of Croatian fauna which has not been registered so far because of lack of molecular identification. Further studies are needed to determine the presence of this mosquito species in the Croatian lowlands.

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