

# FREQUENCY OF *CULEX PAPIENS* COMPLEX MEMBERS IN CONTINENTAL CROATIA

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**Bušić, N., Lukić, D., Mirković, G., Skuzin, I. & Merdić, E.: Frequency of *Culex pipiens* complex members in continental Croatia. Nat. Croat., Vol. 33, No. 1, 1-11, Zagreb, 2024.**

Members of the *Culex pipiens* complex are considered a potential vector in the Republic of Croatia, which is why it is extremely important to have data on its distribution as well as data on the species and biotypes found within the Complex. This study was conducted with the aim of determining the frequency of biotypes within the *Culex pipiens* complex, and especially *Culex pipiens/molestus* hybrids. *Culex pipiens* biotype *pipiens* and *Culex pipiens* biotype *molestus* differ in behavior and physiology. *Cx. pipiens* biotype *pipiens* mainly feed on the blood of birds, while *Cx. pipiens* biotype *molestus* feeds on the blood of mammals. By feeding on both hosts, hybrids can significantly increase the possibility of transmission of various disease agents. The study was conducted in June 2022. Individuals were sampled using a net with a diameter of 25 cm in water habitats and using plastic containers on small and shallow water surfaces. As a diagnostic marker for discrimination among species and biotypes within the *Cx. pipiens* complex, the nuclear ACE2 and CQ11 microsatellite loci were used. Three representatives of the genus *Culex* were confirmed: *Culex torrentium*, *Cx. pipiens* biotype *pipiens* and *Cx. pipiens* biotype *molestus*. The most common taxon in the research area was *Cx. pipiens* biotype *pipiens* (62% in urban and 69% in rural areas). *Cx. torrentium* was recorded in the rural area of north-western Croatia only. *Cx. pipiens* biotype *molestus* was recorded in south-eastern part of continental Croatia only (8.33%), with greater dominance in urban areas. *Cx. pipiens/molestus* hybrid was recorded in rural areas of the south-eastern part of continental Croatia (8.33%). This research with its results represents a platform for future research of the *Cx. pipiens* complex in Croatia.

**Key words:** *Culex pipiens* complex, vectors, biotypes, hybrids, molecular determination

**Bušić, N., Lukić, D., Mirković, G., Skuzin, I. & Merdić, E.: Učestalost članova *Culex pipiens* kompleksa u kontinentalnoj Hrvatskoj. Nat. Croat., Vol. 33, No. 1, 1-11, Zagreb, 2024.**

Članovi *Culex pipiens* kompleksa smatraju se potencijalnim vektorima u Republici Hrvatskoj, zato je od iznimnog značaja imati podatke o njihovoj rasprostranjenosti te podatke o samim vrstama i biotipovima koji se nalaze unutar kompleksa. Ovo istraživanje provedeno je s ciljem utvrđivanja učestalosti biotipova unutar *Cx. pipiens* kompleksa, te posebno *Culex pipiens/molestus* hibrida. *Culex pipiens* biotip *pipiens* i *Culex pipiens* biotip *molestus* razlikuju se u ponašanju i fiziologiji. *Cx. pipiens* biotip *pipiens* se uglavnom hrani krvlju ptica, dok se *Cx. pipiens* biotip *molestus* hrani krvlju sisavaca. Hraneći se na oba domaćina, hibridi mogu značajno povećati mogućnost prijenosa raznih uzročnika bolesti. Istraživanje je provedeno u lipnju 2022. godine. Jedinke su uzorkovane mrežicom promjera 25 cm u vodenim staništima te plastičnim posudama na malim i plitkim vodenim površinama. Kao dijagnostički marker za razlikovanje između vrsta i biotipova unutar *Cx. pipiens* kompleksa korišten je nuklearni ACE2 i CQ11 mikrosatelitni lokus. Molekularnim analizama potvrđena su tri predstavnika roda *Culex*: *Culex torrentium*, *Cx. pipiens* biotip *pipiens* i *Cx. pipiens* biotip *molestus*. Najčešća svojta na području istraživanja bio je *Cx. pipiens* biotip *pipiens* (62% u urbanim i 69% u ruralnim područjima). *Cx. torrentium* zabilježen je samo u ruralnom području sjeverozapadne Hrvatske. *Cx.*

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*pipiens* biotip *molestus* zabilježen je samo u jugoistočnom dijelu kontinentalne Hrvatske (8,33%) s većom dominacijom u urbanim sredinama. *Cx. pipiens/molestus* hibrid zabilježen je u ruralnim područjima jugoistočnog dijela kontinentalne Hrvatske (8,33%). Ovo istraživanje svojim rezultatima predstavlja platformu za buduća istraživanja *Cx. pipiens* kompleksa u Hrvatskoj.

**Ključne riječi:** *Culex pipiens* kompleks, vektori, biotipovi, hibridi, molekularna determinacija

## INTRODUCTION

Because of the various climatic, geological and ecological factors, the territory of the Republic of Croatia comprises many different ecosystems and habitats that foster a great biodiversity of flora and fauna (MERDIĆ, 2020). MERDIĆ *et al.* (2004) published the first list of Croatian mosquitoes, which included 48 species. The list was recently updated and currently contains 52 species of mosquitoes classified into 8 genera, of which the genus *Culex* contains 7 species (MERDIĆ *et al.*, 2020). In general, mosquitoes are ubiquitous organisms with great adaptability and they can be found worldwide on all continents except Antarctica. As a result of climate change and general global warming more and more research is focused on predicting the impact of these changes on species distribution. Short lived ectothermic species including pests, pathogens and vectors are of particular interest (COUPER *et al.*, 2021). Temperature, for example, has a direct influence on the functional properties of mosquitoes, and thus on the dynamics of the population and the dynamics of the disease which they transmit as vectors. (CATOR *et al.*, 2020).

The spread of various species of mosquitoes, especially species from the *Culex pipiens* complex (further in the text, when not emphasized, the word Complex refers to *Cx. pipiens* complex), have crucial importance for public health, considering that they are vectors of many infectious diseases (VINOGRADOVA *et al.*, 2007). According to COLLINS & PASKEWITZ (1996), a species complex is defined as a group of evolutionarily closely related species that are difficult to separate morphologically. Species within the *Cx. pipiens* biotype *pipiens* complex can be separated based on the male genitalia (BARR, 1957; DOBROWORSKY, 1967), but this is not applicable in the females that are of crucial importance regarding their vector roles. The *Cx. pipiens* complex in Europe includes several species and forms *Cx. pipiens* Linnaeus (former *Cx. pipiens* biotype *pipiens*), *Culex pipiens* biotype *molestus* Forskal, *Culex quinquefasciatus* Say and sibling species *Culex torrentium* Martini (BECKER *et al.*, 2020). In Croatia *Cx. pipiens* biotype *pipiens*, *Cx. pipiens* biotype *molestus* and *Cx. torrentium* are present (MERDIĆ *et al.*, 2018; Bušić *et al.*, 2021).

Although the members of the Complex are morphologically very similar and difficult to distinguish regardless of developmental stage, they differ in ecophysiological features (VINOGRADOVA, 2007), which has a great effect on their vector capacity (FA-RAJOLLAHI *et al.*, 2011). *Cx. torrentium* represents separate sibling species because of the genetics distance to *Cx. pipiens* s.l. (BECKER *et al.*, 2020). The common characteristic of the members of the Complex is that they are very weak flyers and therefore will not go far from their breeding sites. Besides the many differences in behavior and physiology within the Complex, the greatest differences are between *Cx. pipiens* biotype *pipiens* and *Cx. pipiens* biotype *molestus* (BECKER *et al.*, 2012), which are also phylogenetically separated (FONSECA *et al.*, 2004; WEITZEL *et al.*, 2009). *Cx. pipiens* biotype *pipiens* conducts winter diapause, is anautogenous (eggs are laid only after a blood meal) and eurygamanous (closed spaces are a problem during mating) (HERBACH, 1984; AMRAOUI *et al.*, 2012). They can breed in various water bodies which can be clean or polluted, as

big as a pond or as small as a puddle. They are found widespread in urban and rural environments (BECKER *et al.*, 2012). *Cx. pipiens* biotype *molestus* does not undergo winter diapause, which means that it is capable of reproducing new individuals throughout the year, which is why we can often find it in cellars in winter (MERDIĆ & VUJČIĆ KARLO, 2005). In addition, unlike *Cx. pipiens* biotype *pipiens*, *Cx. pipiens* biotype *molestus* can lay the first series of eggs without a blood meal (it is autogenous) and can reproduce indoors (stenogamous) (CLEMENTS, 1992; AMRAOUI *et al.*, 2012). Groundwater in urban areas with a high content of organic matter favors the reproduction of *Cx. pipiens* biotype *molestus* (BECKER *et al.*, 2012).

*Cx. torrentium* is an ornithophilic, eurygamous, anautogenous member of the Complex that is in diapause during the winter. It can be found together with *Cx. pipiens* biotype *pipiens* in the warmer months of the year (BECKER *et al.*, 2012). *Cx. quinquefasciatus* is with *Cx. pipiens* s.l. most widespread in temperate and tropical areas (AMRAOUI *et al.*, 2012).

As vectors, they participate in the transmission of arbovirus infections, which have a global effect in increased morbidity and mortality in humans and livestock (WORLD HEALTH ORGANIZATION, 2020). More than 100 different arboviruses are known that affect the development of disease states in humans, and the infection itself can be asymptomatic, but in some cases, more severe forms of the disease develop and can cause hemorrhagic fever or encephalitis, which ultimately result in death (DIAS-BADILLO, 2011). The global spread of West Nile Virus (WNV) has led to the arrival of the virus in the area of Central and Eastern Europe, which can worry the population because of the neuroinvasive diseases that this virus can cause in humans and animals. *Cx. pipiens* complex is considered to be a potential vector in the Republic of Croatia (MERDIĆ, 2013). WNV human infections appeared in 2012 in the eastern part of the Republic of Croatia, and in 2013 it was recorded in northwestern Croatia, specifically in the area of Međimurje, Zagreb County and the City of Zagreb (MERDIĆ *et al.*, 2013; VILIBIĆ ČAVLEK *et al.*, 2014). WNV infections have been continuously present in the human population since 2012 with the biggest outbreak in 2018 when 61 human cases of West Nile neuroinvasive disease were noted in Croatia (VILIBIĆ ČAVLEK *et al.*, 2021). In addition, *Cx. pipiens* complex is a proven vector of Usutu virus (USUV) which was, from its first discovery in Croatia in 2011 (BARBIĆ *et al.*, 2013) and until 2019, continuously present in the area of northwestern Croatia (KLOBUČAR *et al.*, 2021).

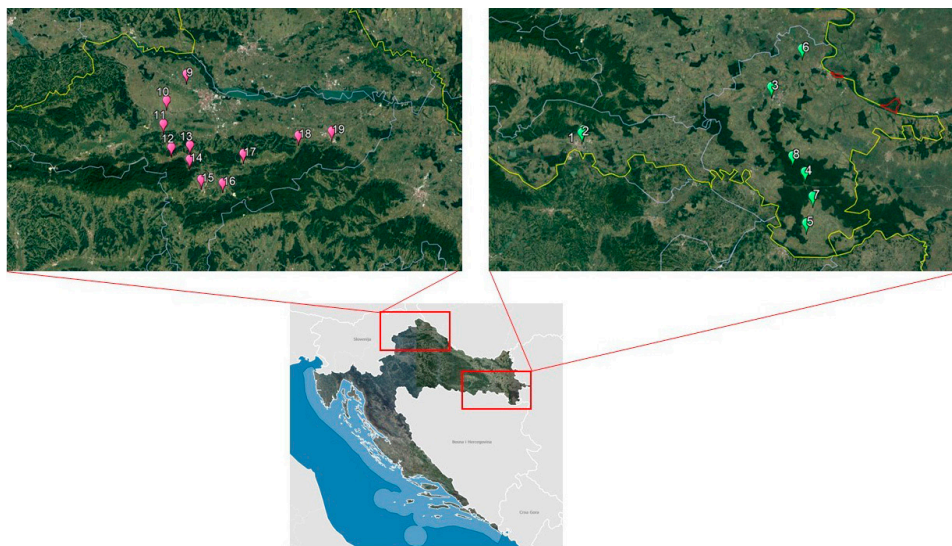
Of great epidemiological importance are the hybrids of *Cx. pipiens* biotype *pipiens* and *Cx. pipiens* biotype *molestus*. Namely, their hybridization is known in the USA and Europe, which is of great importance because their hybridization leads to the creation of opportunists (SPIELMAN, 2001; FONSECA, 2004). *Cx. pipiens* biotype *pipiens* mainly bite birds while *Cx. pipiens* biotype *molestus* feed on mammals. The resulting hybrids feed on both hosts, which increases the possibility of the transmission of various pathogens. Importantly, the risk of mosquitoes becoming vectors of WNV between birds and humans is increasing (FONSECA *et al.*, 2004; GOMES *et al.*, 2013).

Research on mosquitoes in south-east continental Croatia and north-west Croatia was carried out with the aim of determining species and biotypes from the *Cx. pipiens* complex, which are morphologically almost identical, because of which polymorphic DNA regions are used as the main determining element.

## MATERIALS AND METHODS

### Study area

For the purposes of this research, mosquito sampling was carried out in June 2022 at eight locations in south-east continental Croatia (Vukovar-Srijem and Brod-Posavina County) and 11 locations in north-west Croatia (Varaždin County) (Fig. 1). The altitude of the researched area ranged from 78 to 258 meters. In these parts of Croatia, the climate is predominantly continental, with some exceptions. The Vukovar-Srijem County is located in the extreme southeast of the Republic of Croatia. In this area, summers are sunny and hot, and winters are cold and often snowy. The mean annual temperature is 11°C with a mean warmest maximum of 29.9°C and a mean minimum of 12.2°C. Average annual rainfall is lowest in the easternmost part, amounting to around 650 mm. The most rainfall occurs in spring and mid-summer. The average relative humidity is 79%. Brod - Posavina is the southernmost Slavonian county in Croatia. It is located in an area with rarely pronounced meteorological extremes, which is characterized by mean monthly temperatures exceeding 10°C for more than four months, while the mean temperature of the warmest month is below 22°C. We distinguish between the local climate of the Prigorje area and the Prisava plain. The mountainous area is characterized by longer insulation due to protection by mountain ridges, higher rainfall and higher temperatures. The Prisava lowland is characterized by high relative humidity, more frequent occurrences of fog and shorter duration of insolation. Varaždin County is located on the edge of the Pannonian region and is characterized by three relief areas: northern plains, southern and western hills, with mountain massifs. Characteristic of the Peripannonian region are moderately warm summers and rainy and cold winters. The average annual air temperature is 10°C (temperature and precipitation data refer to the ten-year average, Croatian Meteorological and Hydrological Service).



**Fig. 1.** Sampling sites of mosquitos in South East continental Croatia (green marks, 1-8) and in North West Croatia (pink marks, 9-19)

## Sampling and morphological identification

Individuals were sampled by using a net with a diameter of 25 cm in water habitats and using plastic containers on small and shallow water surfaces. After sampling, some individuals were stored in 96% ethanol to preserve the genetic material until analysis and some were left to develop to the adult stage. All mosquitoes were morphologically identified according to GUTSHEVICH *et al.* (1974) and BECKER *et al.* (2020).

## Molecular identification

Three individuals of the *Cx. pipiens* complex from each locality were analyzed for molecular determination. Entire larvae or adult specimens were used. The GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma) was used for extraction of genomic DNA, following the protocol for rodent tail preparation with slight modifications (incubation in Proteinase K overnight; final DNA elution in 100 µl of elution solution). Following SMITH & FONSECA (2004) the nuclear ACE2 microsatellite locus was used as a diagnostic marker for discrimination between sibling species *Cx. pipiens* s.l. and *Cx. torrentium*. To detect *Cx. pipiens* biotypes, as well as their hybrids, the microsatellite locus CQ11 was analyzed by multiplex PCR (BAHNCK & FONSECA, 2006). Results were obtained by analyzing the size and number of DNA fragments visible by gel electrophoresis. For *Cx. pipiens* biotype *pipiens* the expected band size is 200 base pairs, while the band characteristic for *Cx. pipiens* biotype *molestus* is 250 base pairs in size (BAHNCK & FONSECA, 2006). The hybrid form had both bands present on the gel (Fig. 4).

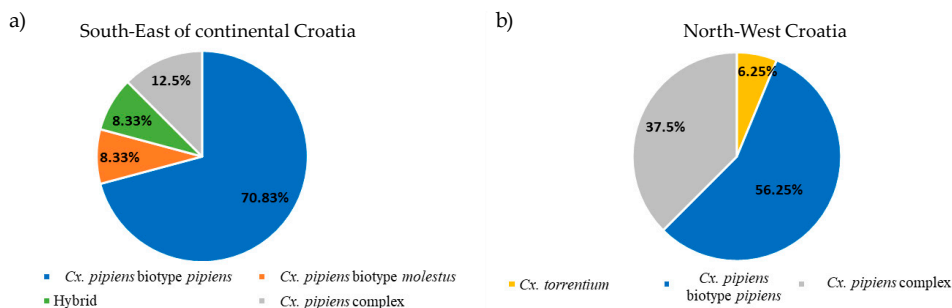
## RESULTS

The morphological was followed by a molecular determination, which confirmed the presence of biotypes and species of the *Cx. pipiens* complex in 16 locations, eight of which are from North West Croatia and eight from the South East of continental Croatia (Tab. 1). It should be noted that nine localities covered urban areas, and the other 10 were from rural ones. A total of 60 mosquito samples, three individuals from each location, were subjected to molecular analyses. Some of the samples did not give an interpretable result and for that reason they were not considered in the final result. Therefore, instead of 60, 40 samples were ultimately left. Three representatives of the mentioned Complex were determined in the samples, sibling species *Cx. torrentium* and both biotypes *Cx. pipiens* biotype *pipiens* and *molestus*. Some samples that could not be detected as biotypes or hybrids were identified only as *Cx. pipiens* complex. The most common taxon in the researched area was *Cx. pipiens* biotype *pipiens* as can be seen from Fig. 2. Regardless of whether it is an urban or rural environment that is concerned, this species is represented by a dominant percentage. For the urban environment this is 62% (13 out of a total of 21 samples), and for the rural environment 69% (13 out of a total of 19 samples) (Fig. 3). In North West Croatia, this taxon comprises 56.25% of the total of 16 individuals that were examined from that region and in the South East of continental Croatia it accounts for 70.83% of a total of 24 individuals. *Cx. torrentium* was recorded in north-west Croatia only with a contribution of 6.25%, in rural areas. *Cx. pipiens* biotype *molestus* was recorded in the area of south-east continental Croatia only. Out of a total of 24 individuals sampled there, two of them were determined as the mentioned biotype, which comes to 8.33% (Fig. 2). If we also consider the locations in the south-east of continental Croatia where this biotype was found,

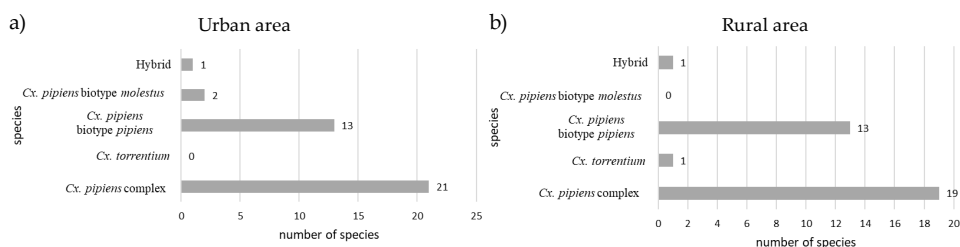
**Tab. 1.** Description of mosquito sampling sites, habitats and species found at the sites.\* **species** detected after molecular analysis with the nuclear ACE2 microsatellite locus\*\* **biotype** or hybrid detected after analysis with microsatellite locus CQ11

Nb.	Sampling site	County	Sampling date	GPS coordinates	altitude/m	Species*	biotype/hybrid**	Type of habitat
1	Slavonski Brod	Brod-Posavina	03.06.2022.	45°10'25" N 18°02'16" E	97	<i>Cx. pipiens</i> s.l.	biotype <i>molestus</i> hybrid	barrel
2	Slavonski Brod	Brod-Posavina	03.06.2022.	45°09'58" N 18°01'57" E	96	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i>	barrel
3	Vinkovci	Vukovar-Srijem	03.06.2022.	45°17'17" N 18°46'20" E	79	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i>	channel
4	Spačva (Donje Novo Selo)	Vukovar-Srijem	03.06.2022.	45°03'15" N 18°54'00" E	85	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i> hybrid	pound
5	Drenovci	Vukovar-Srijem	03.06.2022.	44°54'50" N 18°54'11" E	82	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i>	barrel
6	Otok	Vukovar-Srijem	03.06.2022.	45°09'38" N 18°53'40" E	83	<i>Cx. pipiens</i> s.l.	biotype <i>molestus</i>	barrel
7	Vrbanja	Vukovar-Srijem	03.06.2022.	44°59'13" N 18°55'44" E	80	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i>	barrel
8	Otočki Virovi	Vukovar-Srijem	03.06.2022.	45°05'55" N 18°51'08" E	78	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i>	pound
9	Hrašćica	Varaždin	23.06.2022.	46°19'42" N 16°17'21" E	175	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i>	barrel
10	Vidovec	Varaždin	23.06.2022.	46°17'11" N 16°14'41" E	179	<i>Cx. torrentium</i>	-	barrel
11	Tužno	Varaždin	23.06.2022.	46°14'59" N 16°14'20" E	185	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i>	barrel
12	Završje Podbelsko	Varaždin	23.06.2022.	46°12'47" N 16°15'30" E	194	<i>Cx. pipiens</i> s.l.	-	barrel
13	Sveti Ilija	Varaždin	23.06.2022.	46°13'01" N 16°18'02" E	199	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i>	concrete trough
14	Podevčevo	Varaždin	23.06.2022.	46°11'41" N 16°18'05" E	258	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i>	barrel
15	Novi Marof	Varaždin	23.06.2022.	46°09'49" N 16°19'40" E	188	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i>	channel
16	Ljubešćica	Varaždin	23.06.2022.	46°09'32" N 16°22'37" E	184	none	none	barrel
17	Varaždinske Toplice	Varaždin	23.06.2022.	46°12'19" N 16°25'22" E	182	none	none	barrel
18	Slanje	Varaždin	23.06.2022.	46°14'07" N 16°32'51" E	163	<i>Cx. pipiens</i> s.l.	biotype <i>pipiens</i>	barrel
19	Ludbreg	Varaždin	23.06.2022.	46°14'35" N 16°37'24" E	157	none	none	small boat

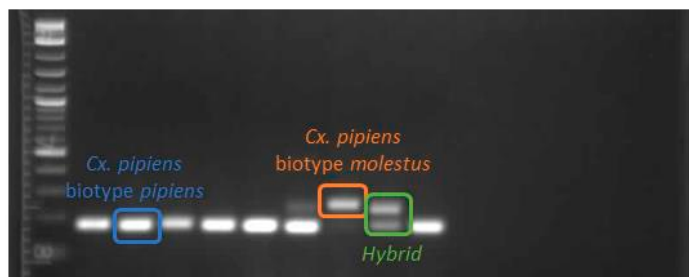
it can be concluded that it is more dominant in urban (10%, 2 individuals out of 21) than in rural areas. In the same part of Croatia hybrid *Cx. pipiens* / *molestus* were recorded in two locations in rural areas and make up 8.33% of the sample (2 individuals out of a total of 24). Not a single individual of *Cx. pipiens* biotype *molestus* or hybrid was detected in north-west Croatia.



**Fig. 2.** Abundance of species in South East continental Croatia (a) and North West (b) Croatia in percentages.



**Fig. 3.** Number of specimens in the urban (a) and rural (b) part of the research area.



**Fig. 4.** Example of PCR amplification of the flanking region of the CQ11 microsatellite of *Culex pipiens* complex collected in South East of continental Croatia.

## DISCUSSION

As previously mentioned, the *Cx. pipiens* complex is made up of several species and forms that are extremely difficult to distinguish and classify at the morphological level. For this reason, in order to fulfill the purpose of this research, deviations at the molecular level between individual components of the mentioned Complex were used. The most abundant taxon in south-east continental Croatia and north-west Croatia was *Cx. pipiens* biotype *pipiens*. As a polyzonal and ecologically plastic species (VINOGRADOVA, 2000) it is widely distributed in the Holarctic region and is found Europe-wide (HESSON *et al.*, 2014), so this result was expected. In previous scientific research, it was recorded that the reproduction of this species takes place by smaller or larger, natural or man-ma-

de water bodies in urban and suburban areas (BECKER *et al.*, 2012), which is congruent with the localities where the mentioned form was found during this research as can be seen from Tab. 1. Unlike the widespread *Cx. pipiens* biotype *pipiens*, *Cx. pipiens* biotype *molestus* has a rather narrow distribution and during this scientific work it was recorded more in urban than in rural areas, which does not deviate from the previously determined data related to this biotype. Females of *Cx. pipiens* biotype *molestus* prefer urban environments for breeding (BECKER *et al.*, 2012). This type of habitat is the most favorable for larvae of this biotype, as indicated by the results obtained in this study. It is a fact that members of *Cx. pipiens* complex can interbreed despite the existence of significant differences in physiology, ecology and behavior (OSORIO *et al.*, 2013). Such a phenomenon indicates that the reproductive isolation among the members of the Complex is not complete and they can produce viable offspring under natural conditions (YURCHENKO *et al.*, 2020). This study also determines the presence of hybrids in samples collected in different regions of Croatia. Since *Cx. pipiens* biotype *molestus* was mostly recorded in urban areas, it could be assumed that the *Cx. pipiens/molestus* hybrid form would be more frequent there because there is a higher probability for hybridization to occur due to the higher prevalence of the biotype *molestus* than in rural areas. This is supported by the fact that this form is especially prevalent in cities, as indicated by the attached results, where the circulation of people is high. Interestingly, all hybrid forms of these two biotypes were recorded exclusively in south-east continental Croatia in the lowland area, and not a single sample came from the north-western part of Croatia (higher altitude and lower temperature). If we consider other scientific papers dealing with research into hybrids a similar pattern of incidence can be observed. Namely, RUDOLF *et al.* (2013) determined that the frequency of hybridization between the *Cx. pipiens* biotype *pipiens* and *Cx. pipiens* biotype *molestus* gradually increases from north to south Germany which, as in the case of Croatia, may be related to the climatic factors of the sampling area, but to confirm this, further studies are needed. Evidence of hybridization can be of great public health importance if their vector role is considered because they are the primary vectors of WNV and USUV in Europe (FROS *et al.*, 2015; VILIBIC-CAVLEK *et al.*, 2020). The viremia of WNV in humans and other mammals is not high enough for the virus to be transmitted from person to person through mosquitoes, but hybrid forms *Cx. pipiens/molestus* can potentially cause the spread of WNV among humans (FONSECA *et al.*, 2004). The *Cx. torrentium* was detected exclusively in the north-western part of Croatia in rural areas. If we consider the geographical, and consequently the climatic differences between the north-western and south-eastern parts of Croatia, this result is consistent with the already known data for this species. Previous works indicate the presence of this species at higher altitudes such as Velebit and Gorski kotar (Bušić *et al.*, 2021a; 2021b) and the results of this study are in line with previous research. In the north-western part of Croatia, samples were collected at higher altitudes than in the east. Therefore, since *Cx. torrentium* is normally dominant in the north of Europe, a higher altitude is more suitable for this species because of the lower temperatures.. It should also be mentioned that *Cx. torrentium* has been identified as a potential vector for WNV in central and northern Europe (JANSEN *et al.*, 2019).

So far, only a few studies related to *Cx. pipiens* complex have been conducted in Croatia and none of them deal entirely with the form of the Complex. Considering the possible public health significance of species, biotypes and hybrids within the Complex, this research provides a platform for the further research that is urgently needed.



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