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Doctoral Study of Enviromental Protection and Nature Conservation

Nataša Bušić, mag. educ. biol. et chem.

BARCODING OF CROATIAN MOSQUITO (DIPTERA, CULICIDAE) FAUNA AND POPULATION GENETICS OF THE *CULEX PIPIENS* COMPLEX

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Supervisor: *Enrih Merdić, PhD, Full Professor, Department of Biology, Josip Juraj Strossmayer University of Osijek*
Branka Bruvo Mađarić, PhD, Senior Research Associate, Ruđer Bošković Institute, Zagreb

Summary

The global spread of arthropod-borne diseases, particularly by mosquitoes (Diptera: Culicidae), emphasizes the need for accurate and precise species identification, which is crucial for effective vector surveillance and control strategies. The main aim of this six-year study was to develop the first comprehensive DNA barcode reference library for the Croatian mosquito fauna. Samples were collected in three biogeographical regions of Croatia — the Pannonian-Peripannonian, the central mountainous, and the Mediterranean region — using different trapping methods. The specimens were morphologically identified, followed by molecular analysis using the mitochondrial COI gene, the nuclear ITS2 region (for the *Anopheles maculipennis* complex) and the microsatellite markers ACE2 and CQ11 (to distinguish the biotypes of *Culex pipiens* s. l. and the species *Culex torrentium*). A total of 405 specimens representing six genera were analyzed. When delimiting the species, the COI sequences were assigned to 31 MOTUs with BIN-RESL, 30 with bPTP and 28 with ASAP, which is largely consistent with the morphology. However, COI showed limitations in distinguishing morphologically similar or complex species within the genera *Culex, Aedes* and *Anopheles*. A total of 34 COI barcodes and ITS2 sequences for three *Anopheles* species were added to the national database. Notably, a specimen of the Intrudens group (*Aedes intrudens*) represents the first record of this species for Croatia. Eight new species were discovered for the central mountainous region, including invasive species *Aedes albopictus* (in Lika). Diversity indices (Jaccard, Shannon-Wiener, Simpson, Evenness) showed significant differences in mosquito diversity and abundance depending on altitude and habitat. The biotypes of *Cx. pipiens* were identified for the first time in Croatia, and *Culex torrentium*, which was previously thought to occur only at high altitudes in southern Europe, was also found at lower altitudes, contributing to new insights into its ecological distribution.

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BARKODIRANJE FAUNE KOMARACA (DIPTERA, CULICIDAE) HRVATSKE I POPULACIJSKA GENETIKA POLITIPSKE VRSTE *CULEX PIPIENS* KOMPLEKS

Nataša Bušić, mag. educ. biol. et chem.

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Mentor/komentor: *prof. dr. sc. Enrih Merdić, Odjel za biologiju, Sveučilište J. J. Strossmayera u Osijeku*
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Sažetak doktorskog rada:
Globalno širenje bolesti koje prenose člankonošci, posebice komarci (Diptera: Culicidae), naglašava potrebu za točnom i preciznom identifikacijom vrsta, što je ključno za učinkovit nadzor vektora i razvoj strategije kontrole komaraca. Glavni cilj ovog šestogodišnjeg istraživanja bio je razviti prvu sveobuhvatnu referentnu biblioteku DNK barkodova za hrvatsku faunu komaraca. Uzorkovanje je obavljeno u tri biogeografske regije Hrvatske - panonsko-peripanonskoj, srednje planinskoj i mediteranskoj - različitim metodama hvatanja. Nakon morfološke identifikacije uslijedile su molekularne analize uzoraka pomoću mitohondrijskog COI gena, ITS2 regije nuklearnog ribosomalnog operona (za vrste unutar *Anopheles maculipennis* kompleksa) i mikrosatelitnih markera ACE2 i CQ11 (za razlikovanje biotipova vrsta *Culex pipiens* s. l. i vrste *Culex torrentium*). Za potrebe barkodiranja, analizirano je ukupno 405 jedinki iz šest rodova. Prilikom razgraničenja vrsta, COI sekvence dodijeljene su u 31 MOTU s BIN-RESL, 30 s bPTP i 28 s ASAP metodama, koje su se u velikoj mjeri poklapale s morfologijom. Međutim, COI je pokazao nedovoljnu razlučivost kod morfološki sličnih vrsta te kompleksa vrsta unutar rodova *Culex, Aedes* i *Anopheles*. U nacionalnu bazu podataka uključena su ukupno 34 COI barkoda i ITS2 sekvence za tri vrste roda *Anopheles*. Jedan uzorak unutar grupe Intrudens (*Aedes intrudens*) predstavlja prvi nalaz ove vrste u Hrvatskoj. U središnjem planinskom području otkriveno je osam novih vrsta za to područje, uključujući invazivnu vrstu *Aedes albopictus* (u Lici).Indeksi raznolikosti (Jaccard, Shannon-Wiener, Simpson, Evenness) pokazali su značajne razlike u raznolikosti i brojnosti komaraca ovisno o nadmorskoj visini i staništu. Biotipovi vrste *Cx. pipiens* molekularno su identificirani po prvi puta u Hrvatskoj, a vrsta *Culex torrentium*, za koju se dosad smatralo da se javlja samo na višim nadmorskim visinama u južnoj Europi, pronađena je i na nižim nadmorskim visinama, što je pridonijelo novom uvidu u njezinu ekološku rasprostranjenost.

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2. *dr.sc. Martina Podnar Lešić, viša znanstvena suradnica, član*
3. *dr. sc. Martina Pavlek, znanstvena suradnica, član*
4. *doc. dr. sc. Anita Galir, docentica, zamjenski član*

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I dedicate this dissertation to them: Marija and Stjepan Graovac.

"I can do all this through Him who gives me strength. Phil 4:13"

Contents

GENERAL INTRODUCTION

Mosquitoes (Culicidae), an insect family from the order Diptera, are among the most widespread and successful organisms, as they can adapt to a variety of terrestrial habitats with the exception of deserts and permanently frozen regions (Becker et al., 2020). Over 3,700 mosquito species have been described worldwide (Mosquito Taxonomic Inventory, 2024). From a public health perspective, mosquitoes are the deadliest and most studied group of insects in the world, as they are the main vectors of numerous diseases that affect both humans and animals. They transmit serious diseases such as Zika, West Nile, chikungunya, dengue and malaria. Some cases are mild, others can be life-threatening. Given their medical importance, the correct identification of mosquito species is crucial for effective vector surveillance, disease control and the implementation of targeted management strategies. However, the identification of mosquitoes is often challenging. Traditional taxonomic keys are based on the external morphological characteristics of adult females and fourth-stage larvae (Weeraratne et al., 2018). However, these features can be lost or damaged during collection (e.g. wings, legs, scales), which complicates the process. In addition, species complexes with almost identical morphological characteristics make accurate identification difficult (Beebe, 2018). This situation is exacerbated by a significant decline in the number of taxonomists and trained identification experts (Utsugi et al., 2011). Even experienced experts often find it difficult to distinguish species based on morphology alone, highlighting the growing need for alternative methods that are fast, reliable and applicable across all life stages and specimen conditions. The urgency of such methods is further emphasised by the increasing interest in biodiversity and its importance for ecology, evolutionary biology, environmental protection, agriculture and the economy. In addition, predicting the effects of climate change and global warming on the distribution of species has become an important area of research. This is especially true for short-lived ectothermic organisms such as insects, parasites and vectors, which are particularly sensitive to environmental changes (Couper et al., 2021). Advances in DNA sequencing technology have enabled scientists to perform simple, inexpensive and rapid DNA analysis, by taking samples from the entire mosquito, the legs, the wings or another part of the insect's body (Utsugi et al., 2011; Murugan et al., 2016). Hebert et al. (2003) introduced DNA barcoding and proposed a method based on sequencing a standardized fragment of the mitochondrial gene for cytochrome oxidase subunit I (COI) as a rapid and reliable tool for identifying a wide range of biological samples. The mitochondrial genome has been shown to be a better tool for species identification than the nuclear genome due to its haploid inheritance, lack of introns and limited recombination (Hebert et al., 2003). The COI gene is recognized as a good genetic marker for species differentiation due to two major factors: 1. universal primers specifically amplify the targeted part of the COI gene in almost all animal species, 2. the rate of evolutionary changes of this gene allows differentiation of closely related species (Wang et al., 2012). Among all barcoded insect groups, mosquitoes are the most intensively barcoded, probably due to their important role as vectors (Weeraratne et al. 2018). Many studies have shown that COI is a valid molecular marker for mosquito species identification and cryptic species recognition (Adeniran et al., 2021; Laurito et al., 2021; Viveros-Santos et al., 2022; Chaiphongpachara et al., 2022; Lamichhane et al., 2024). In addition to the COI gene, the region of the nuclear ribosomal DNA spacer ITS2 (Internal Transciber Spacer 2) is also a frequently used genetic marker in mosquito identification, especially to distinguish species within complexes where COI is not sufficiently variable (Weeraratne et al., 2018; Khoshdel-Nezamiha et al., 2016; Shahhosseini et al., 2020; Lamichhane et al., 2024). The use of other genetic markers and the combination of data sets can increase the accuracy of identification.

The Barcode of Life Data System (BOLD) is a bioinformatics platform that supports the entire DNA barcoding process, from sample collection to the creation of a validated barcode library. It fulfills three main functions: data archive (it stores sample and sequence dataset for barcoding studies), analysis tool (it helps manage, assure quality, and analyze barcoding data), and collaboration platform (it enables collaboration between research communities through secure data entry and web-based access) (Ratnasingham and Hebert, 2013). BOLD was originally developed as a dedicated DNA barcoding facility, but has evolved into a resource for the global DNA barcoding community, supporting large-scale barcoding campaigns for species such as birds, fish and Lepidoptera. The system also facilitates the submission of DNA barcoding data to other public databases and repositories such as NCBI once the results are released to the public. In addition, BOLD periodically retrieves the public COI barcoding data from NCBI and assigns these sequences with BOLD-ID numbers, making it easier for users to get an overview of all currently available DNA barcoding data, regardless of the originally submitted database. BOLD uses established algorithms within the Barcode Index Number (BIN) system to group sequences into operational taxonomic units that closely match species, according to the existance of the so-called barcoding gap, with each new cluster assigned a globally unique identifier (BIN number) (Ratnasingham and Hebert, 2013). Recent DNA barcoding studies in Europe (Engdahl et al., 2014; Versteirt et al., 2015; Talaga et al., 2017;

Muja-Bajraktari et al., 2019; Hohmeister et al., 2021; Deblauwe et al., 2021; Madeira et al., 2021; Bakran-Lebl et al., 2022; Kirik et al., 2022; Chaiphongpachara et al., 2022) have established national DNA barcode libraries for several countries. These efforts have led to the discovery of new species, the confirmation of previously unrecorded species in certain regions and the identification of cryptic taxonomic units (Lilja et al., 2018). Nevertheless, the amount of barcode sequences for European mosquitoes with accurate country designation and species identification is still limited and accounts for less than 10 % of the total Culicidae records in BOLD.

With its geographical location in the Western Balkans and the Mediterranean region, Croatia is one of the so-called "biodiversity hotspots" in Europe and worldwide, due to the diverse geological past of the region, the different composition of habitats and the unique climatic and hydrological conditions. The country is located in south-eastern Europe, in the middle of the northern temperate zone; it consists mainly of lowland areas (below 200 m above sea level), which make up 53.4 % of the territory, while 25.6 % are hilly regions (200 - 500 m a. s. l.) and 21.0 % are mountainous areas (over 500 m a. s. l.). According to Bertić et al. (2001) Croatia is divided into three biogeographical regions, the Pannonian-Peripannonian region in the north and east, the central mountainous region in the middle and the Mediterranean region in the south, and this classification is used in this dissertation. The climate varies throughout the country, with a predominantly temperate continental climate, a montane climate at higher altitudes and a Mediterranean climate along the coast and in parts of the hinterland.

Mosquito populations in Croatia have been well studied in certain areas, such as Slavonia (Merdić et al., 2010), Dalmatia (Romanović and Merdić, 2011), Istria (Merdić et al., 2008), and some other regions (Merdić, 1995a; Merdic et al., 1995b). To date, 52 mosquito species have been identified in Croatia (Merdić et al., 2020), including two invasive species: *Aedes albopictus* and *Aedes japonicus* (Klobučar et al., 2006; Klobučar et al., 2018). In the past, most research relied on morphological identification, but more recent studies have used molecular methods to verify the presence of specific species. *Culex torrentium* was molecularly confirmed as part of the Croatian mosquito fauna for the first time in 2018 (Merdić et al., 2018), along with the molecular identification of several other *Anopheles* species (Vignjević, 2014).

Mosquito-borne diseases are usually associated with tropical and subtropical regions, but due to global trade and travel, cases are increasingly reported in Europe as well (Lindh et al., 2018; Martinet et al., 2019; Bakonyi et al., 2020; Lazzarini et al., 2020). Invasive mosquito species like *Aedes albopictus* and *Aedes aegypti*, known vectors of viruses such as dengue, chikungunya, and Zika, are easily spreading. In Croatia, *Aedes albopictus* is well established

(Merdić et al., 2020), though *Aedes aegypti* has not yet been recorded. In Croatia, autochthonous dengue cases first appeared in 2010 (Gjenero-Margan et al., 2011) and have recently re-emerged on Dugi Otok Island (Medić et al., 2025). The outbreaks were also reported in France, Spain and Italy (Riccò et al., 2022; Brem et al., 2023; ECDC 2025).

Malaria has long been considered the most serious mosquito-borne infectious disease in humans (Rougeron et al., 2022). The disease used to be widespread in Europe, but since 2015 the WHO has categorised the European Region as malaria-free and no deaths have been reported in the entire period 2000 - 2023 (WHO, 2024). Recently, however, the number of indigenous transmissions has increased again due to climate change and increased tourist travel to areas with malaria (Kampen et al., 2003; Doudier et al., 2007; Santa-Olalla Peralta et al., 2010; ECDC 2024). According to the latest available annual epidemiological report on malaria from 2022, 13 confirmed cases were reported in the EU (seven in France, three in Germany, two in Spain and one in Ireland) (ECDC, 2024). The most important malaria vector in Europe is *Anopheles maculipennis*, Meigen, 1818 (Sinka et al., 2010). Within the *Anopheles maculipennis* complex, several species are considered dominant vectors of malaria parasites in Europe: *Anopheles atroparvus*, *Anopheles labranchiae*, *Anopheles messeae* and *Anopheles sacharovi* (Sinka et al., 2010; Meibalan et al., 2017). Malaria was officially eradicated in Croatia in 1964, and today only imported cases occur, mainly among migrant workers and returning seafarers, and less frequently among tourists from African and Asian countries (Mulić, 2012; Zhao et al., 2016; Mulić et al., 2021). Despite significant eradication efforts, factors such as climate change, population movements, and the continued presence of effective malaria vectors have increased the likelihood of the re-emerging and spreading of the disease in areas where it was previously eliminated (Rossati et al., 2016; Kulkarni et al., 2022).

One of the most diverse genera of mosquitoes (Diptera: Culicidae) is *Culex* Linnaeus, which comprises 821 species (Harbach, 2013). The main vectors of pathogens that infect animals and humans (dengue virus, Japanese encephalitis virus, Ockelbo virus, Rift Valley fever virus, Saint Louis encephalitis virus and West Nile virus) are considered to be mosquito taxa belonging to the *Culex pipiens* complex (*Cx. pipiens* s. l.) (Vogels et al., 2017). Given the risk of disease outbreaks associated with environmental change and global warming, problems with *Culex* mosquitoes are particularly pronounced (Harbach, 2012). The most widely distributed species within the *Culex* taxa in Central Europe are *Culex torrentium* Martini, 1925 and *Cx. pipiens* s. l. Linnaeus, 1758 with the forms *Cx. pipiens* biotype *pipiens* Linnaeus, 1958 and *Culex pipiens* biotype *molestus* Forskal, 1775. Four other taxa are included within the *Cx. pipiens* complex: *Cx. pipiens pallens* Coquillet, as well as the non-European species *Cx.*

australicus Dobrotworsky and Drummond, *Cx. globocoxitus* Dobrotworsky and *Cx. quinquefasciatus* Say (Farajollahi et al., 2011). *Cx. quinquefasciatus* is with *Cx. pipiens* s. l. most widespread in temperate and tropical areas (Amraoui et al., 2012). Nevertheless, the detection of this species in Turkey (Morcicek et al. 2018), as well as of *Cx. pipiens*/*quinquefasciatus* hybrids in Greece (Shaikevich et al., 2014) could indicate a northward shift in the distribution range of *Cx. quinquefasciatus* in Europe (Becker et al., 2020).

Culex pipiens biotypes are morphologically indistinguishable, but differ in host preference, behavior and habitat (Brugman et al., 2018; Becker et al., 2020; Haba and McBride, 2022), which has a great effect on their vector capacity (Farajollahi et al., 2011). *Culex pipiens* biotype *pipiens* shows eurygamy, anautogeny and heterodynamism. It is mainly active above ground and is classified as ornithophilous (Haba and McBride, 2022). The larvae are usually found in clean waters, including artificial and natural water bodies (Becker et al., 2020). The *Culex pipiens* biotype *molestus* is stenogamous, autogenous and homodynamic. The larvae are commonly found in organically enriched water, often underground in sewage systems and underpasses or in flooded areas, and they choose ecosystems that have been impacted by humans (Becker et al., 2020). In northern latitudes, it feeds on mammals, including humans (Haba and McBride, 2022). The opportunistic feeding behavior of hybrids between *Cx. pipiens* biotype *pipiens* and biotype *molestus* indicates that they can serve as important bridge vectors for the transmission of WNV from infected birds to humans, as has been demonstrated in outbreaks in the United States (Huang et al., 2009). For this reason, their identification is of great importance.

Culex torrentium has almost identical biological and morphological characteristics to the *Culex pipiens* biotype *pipiens* (Becker et al., 2020) and it is therefore difficult to distinguish between these two taxa. The morphology of the male hypopygium can be used to distinguish between *Cx. pipiens* and *Cx. torrentium*, while the females of both species collected in the wild are morphologically identical (Rudolf et al., 2013). The status of this species has not yet been definitively clarified: some consider it a sibling species of the *Cx. pipiens* complex (Becker et al. 2020), and for some, *Cx. pipiens* s. l. and *Cx. torrentium* belong to different subgroups within the subgenus *Culex (Culex pipiens* is assigned to the subgroup *Pipiens,* whereas *Culex torrentium* is placed in the subgroup *Trifilatus)* (Harbach et al., 2017). According to classification of Becker et al. (2020), these two species was treated as sibling species in the work of this dissertation. Some studies have shown that *Culex torrentium* has a significantly higher vector competence for West Nile virus (WNV) and Sindbis virus (SINV) than *Cx. pipiens pipiens* (Hesson et al., 2015; Jansen et al., 2019; Jansen et al., 2023), and is also recognized as an effective vector of Usutu virus (USUV) (Holicki et al., 2020). West Nile virus (WNV) outbreaks have occurred in several European countries (Petrić et al., 2017; Busquets et al., 2019; Bakonyi et al., 2020; ECDC 2018), with Croatia reporting its first clinical cases in 2012 (Pem-Novosel et al., 2014). Recently, Vignjević et al. (2023) proved WNV (lineage 2) for the first time in the mosquito *Culex pipiens* s. l. in Croatia. Likewise, recent studies have shown that USUV became endemic in the northwest Croatia (Klobučar et al., 2021).

Standard mosquito identification markers such as COI and ITS2 showed insufficient resolution to distinguish between very closely related taxa and biotypes within the *Cx. pipiens* complex as well as within some other mosquito species groups. As a result, other molecular markers such as CQ11 microsatellite loci system were developed for specific purposes (Smith and Fonseca, 2004; Bahnck and Fonseca, 2006; Miaoulis et al., 2018).

The main goal of this dissertation is to provide insight into the genetic diversity and geographic distribution of mosquito species in all three biogeographical regions of Croatia using the DNA barcoding. Furthermore, the aim is to determine the effectiveness of molecular methods of mosquito identification and to study the phylogenetic relationships of populations based on the obtained sequences and compare them with those already existing in the BOLD database. On the basis of molecular analyses, this work aims to define taxa of morphologically indistinguishable sister species or species within the complexes (e.g. C*ulex pipiens* and *Anopheles maculipennis* complex). Special emphasis will be on the population genetics of the *Culex pipiens* complex in Croatia and research on distribution of its forms and hybrids.

The dissertation establishes the following hypotheses:

1. DNA barcoding will confirm the presence of recorded mosquito species in Croatia and possibly identify the presence of some additional, previously unrecorded species that are difficult to distinguish morphologically.
2. Phylogenetic analyzes of species populations from the *Anopheles maculipennis* complex based on ITS2 genetic marker will allow the differentiation of sister species within the complex and confirm the previously established low intra-specific variability of the ITS2 region.
3. Comprehensive molecular analyses will confirm the presence of sister species, biotypes and hybrids within the *Cx. pipiens* complex in Croatia.
4. The frequency of *Cx. pipiens* biotype *pipiens* is significantly higher in rural areas, while the frequency of *Cx. pipiens* biotype *molestus* and *Cx. pipiens* biotypes *pipiens/molestus* hybrids is significantly higher in urban areas.

CHAPTER 1

Diversity of mosquito fauna (Diptera, Culicidae) in higher-altitude regions of Croatia

Diversity of mosquito fauna (Diptera, Culicidae) in higher-altitude regions of Croatia

Nataša Bušić[1], Mladen Kučinić[2], Enrih Merdić[1], and Branka Bruvo-Mađarić[3]✉

[1]*Josip Juraj Strossmayer University of Osijek, Department of Biology, Osijek, Croatia*
[2]*University of Zagreb, Faculty of Science, Department of Biology, Zagreb, Croatia*
[3]*Ruđer Bošković Institute, Division of Molecular Biology, Zagreb, Croatia, bruvo@irb.hr*

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ABSTRACT: Global climate change and the accompanying rise in temperature could affect the biology and ecology of a number of vectors, including mosquitoes. High altitude areas that were previously unsuitable for the spread of mosquito vector populations could become suitable. The aim of this research was to study the distribution of mosquito species in higher altitude regions of Croatia. Samples were collected in three areas: Slavonian Mountains, Gorski Kotar, and Middle Velebit. Specimens were morphologically determined and confirmed by DNA barcoding and other genetic markers and showed the presence of 16 species belonging to six genera. The most abundant species were the *Culex pipiens* complex with 50% of the collected specimens. Both *pipiens* (Linnaeus, 1758) and *molestus* (Forskal, 1775) biotypes and their hybrids were identified within the complex, followed by *Culex torrentium* (Martini, 1925) (20.2%), *Culiseta longiareolata* (Macquart, 1838) (8.5%), and the invasive species *Aedes japonicus* (Theobald, 1901) (7.8% of the total number of collected specimens). The remaining 12 species made up 14.7% of the collected specimens. Intraspecific COI p-distances were within the standard barcoding threshold for OTUs, while interspecific genetic distances were much higher, confirming the existence of barcoding gaps. Mosquito fauna of Croatian mountains showed a moderate variety and made 30.8% of the total number of recorded mosquito species in Croatia thus far. ***Journal of Vector Ecology* 46 (1): 65-69. 2021.**

Keyword Index: Mosquito diversity, species complex, COI, microsatellite loci, ITS2.

INTRODUCTION

Given their adaptative mechanisms, mosquitoes are capable of thriving in almost all kinds of environments and various types of habitats. They are disseminated throughout the world, being most numerous in areas with abundant stagnant water, since water is necessary for their development. In mountainous regions, there are fewer mosquito species and their populations are generally smaller, primarily because of the lack of stagnant water that could serve as larval habitats, lower temperature, strong winds, and a large amount of precipitation. Despite these conditions, mosquitoes in the mountains are still present (Merdić et al. 2018, Muja-Bajraktari 2019).

Some species of mosquitoes occupy habitats at various altitudes, including *Anopheles claviger* found up to 2,000 m, *Anopheles maculipennis* up to 2,300 m, *Anopheles plumbeus* up to 2,000 m, *Aedes japonicus* up to 1,200 m (Lake et al. 2016), and *Aedes cataphylla* and *Aedes punctor* up to 2,300 m (Eisen et al. 2008). *Culex torrentium* is known as a species well adapted to cold habitats (Hesson et al. 2014) that appears at high altitudes, with males caught at 1,500 m in the Pyrenees, together with *Culex pipiens* complex males (Hesson et al. 2014).

The mosquito fauna in Croatia has been well explored in Slavonia (Merdić et al. 2010), Dalmatia (Romanović and Merdić 2011), Istria (Merdić et al. 2008), and some other regions (Merdić 1995a, Merdić et al. 2020). However, there is little information on the diversity of mosquito fauna in Croatian mountains (Merdić 1995b, Janssen et al. 2020). Mountains make up a significant part of the Croatian territory, although there are no regions of more than 2,000 m Higher altitude areas can be divided into the Pannonian part of the mountains in the north and the Dinarides mountain chain in the south, which are considered to be the eastern extension of the Alps. Faunal research in mountainous regions in Croatia was conducted in 1995 in the area of Papuk Mountain (highest recorded altitude of 728 m) (Merdić 1995b), where twelve species of mosquitoes have been recorded. More recently, the presence of *Cx. torrentium* was confirmed in the areas of Papuk, Gorski Kotar, and Middle Velebit (Merdić at al. 2018), as well as the spread of the invasive species *Ae. japonicus* (Janssen et al. 2020).

Global climate change and various anthropogenic impacts may have an increasing effect on vector distribution and propagation in temperate climates (Weaver and Reisen 2010). Also, naturally unsuitable habitats in high areas may become more suitable for their development and spread. Continental Europe has faced invasion by exotic aedine mosquito species over the past 30 years, and it can be assumed that these species will probably spread further (Schaffner at al. 2013., Medlock et al. 2015, Petrić et al. 2014, Koban et al. 2019). Two of the invasive mosquitoes, *Ae. japonicus* and *Aedes albopictus,* are widespread throughout Croatia (Klobučar et al. 2006, 2019, Janssen et al. 2020, Merdić et al, 2020).

Identification of mosquito species is mainly done on the basis of morphological characteristics. This has proven to be problematic because diagnostic morphological features are often damaged during sample collection and storage, or are not present in all developmental stages.

Morphological determination is even less appropriate given the fact that mosquitoes often occur in species complexes. Therefore, accurate mosquito identification is an important part of developing vector control strategies, and molecular techniques are frequently used to complement the taxonomical identification of mosquito species (Chan et al. 2014). DNA barcoding is a fast and reliable technique for identifying a wide range of biological samples (Hebert et al. 2003, Kadarkarai et al. 2016). Many studies have shown that the mitochondrial gene for cytochrome oxidase, subunit I (COI) is a valid molecular marker for identifying mosquito species and detecting cryptic species (Hernández-Triana et al. 2019, Adenirana et al. 2021). COI and the nuclear Internal Transciber Spacer region 2 (ITS2) are the most commonly used genetic markers in DNA mosquito barcoding, but for discrimination of very closely related taxa and biotypes within *Cx. pipiens* complex, as well as within some other mosquito species groups, these standard markers are not informative enough. Consequently, various microsatellite loci systems have been recently established for particular purposes (Smith and Fonseca 2004, Bahnck and Fonseca 2006, Miaoulis et al. 2018).

The aim of our study was to determine the presence and genetic diversity of mosquito species at higher altitude regions in Croatia. Characteristic climatic, hydrological, and geographical features make this area specific. Therefore, these data may contribute to a better understanding of the possible spread of mosquito vectors and identification of epidemiologically important species.

MATERIALS AND METHODS

Study area

Sample collection was carried out during July and August, 2017 in three higher altitude areas in Croatia: Slavonian Mountains, Gorski Kotar, and Middle Velebit. Ecological characterization of these areas revealed that they make suitable habitats for mosquitoes (Rauš et al. 1985).

The first area (area 1) included the mountains of Papuk and Požeška Gora. The altitude at which we sampled ranged from a minimum of 113 m to a maximum of 515 m. Papuk is one of the oldest mountains in Slavonia with a specific geological past. Because of the relief and moderate climate with a higher precipitation level than in the rest of Slavonia, there are numerous streams. Plenty of standing water (pools, small ponds, puddles) provides a favorable habitat for the development of mosquito larvae. Most of this area is dominated by forests and small villages where cattle breeding provides favorable places for the development and feeding of mosquitoes.

The second area (area 2) is located in Gorski Kotar. The sampled area ranged from a minimum altitude of 234 m to a maximum altitude of 1,011 m. Gorski Kotar is characterized by a mountain climate with peaks up to 1,500 m. The vegetation depends on the altitude, but coniferous forests mostly predominate. Throughout the forest range there are numerous holes in the ground (from the roots of fallen trees) suitable as larval mosquito habitats.

The third area (area 3) is located in Middle Velebit. The sampled area ranged from the lowest altitude of 529 m to the highest altitude of 1,012 m. The peak of the mountain is a contact zone of two different climate types, Mediterranean and Continental, causing unpredictable weather conditions. The Velebit area is a mosaic of diverse habitats: forests, lawns, rocks, rivers, and rare aquatic habitats. Hollows in limestone rocks, ponds, and dry streams are suitable habitats for mosquito larvae. In the absence of natural habitats, mosquito larvae develop in barrels filled with water, found in almost every garden of this area.

Adult and larval stages were sampled at 68 localities, once per locality: 24 in Papuk, 24 in Gorski Kotar, and 20 in Middle Velebit.

Sampling

Adult mosquitoes were caught using dry ice-baited CDC traps and BG Sentinel traps with their usual attractant, BG Lure, coupled with the dry ice. Larvae were caught individually using a plastic glass or using a 25 cm diameter net with a dipper. Larvae were collected from ponds, canals, barrels, vases in cemeteries, and tires containing rainwater. Some of the collected larvae were brougFht to the laboratory and left for emergence and some were mounted in Canada balsam. Adult mosquitoes were killed using potassium cyanide (KCN) and mounted on entomological pins or stored in 96% ethanol at -20° C for molecular analyses.

Morphological identification

In male mosquitoes of the *Cx. pipiens* complex, genitalia were removed and incubated in hot KOH for 10 min, placed on a microscope slide in a drop of Canada balsam, and covered with a coverslip. Two keys were used for the morphological identification of the specimens: Becker et al. (2010) and Gutsevich et al. (1974).

Molecular identification

Genomic DNA was extracted from a single leg, entire adult, or larva, using the GenEluteTM Mammalian Genomic DNA Miniprep Kit (Sigma), following the protocol for rodent tail preparation with slight modifications (incubation in Proteinase K overnight; final DNA elution in 100 µl of elution solution). Voucher specimens are deposited at the Josip Juraj Strossmayer University of Osijek, Department of Biology, Laboratory of Entomology.

The nuclear ACE2 microsatellite locus was used as a diagnostic marker for discrimination between the sister species *Cx. pipiens* and *Cx. torrentium* (Smith and Fonseca 2004). Samples confirmed as *Cx. pipiens* complex were further analyzed by multiplex PCR for CQ11 microsatellite locus in order to discern between two forms of the species *Cx. pipiens*: f. *pipiens* and f. *molestus*, as well as their hybrids (Bahnck and Fonseca 2006). For the specimens of *An. maculipennis* complex, the ITS2 genomic region was amplified using primers 5.8S (forward) and 28S (reverse) (Di Luca et al. 2004). For all other specimens, the standard barcoding region of mitochondrial COI gene (Hebert et al. 2003) was amplified using universal primers LCO1490 and HCO2198 (Folmer et al. 1994).

Amplification mixtures for COI and ITS2 PCR reactions comprised 1 x DreamTaq™ reaction buffer with 2 mM $MgCl_2$ (Thermo Scientific), 0.2 mM dNTP mix (Qiagen), 0.5 µM each primer, 1.0 U DreamTaq polymerase (Thermo Scientific) and 3 µl DNA in 20 µl reaction volume. COI and ITS2 PCR products were purified and bidirectionally sequenced in Macrogen Inc. (Amsterdam, The Netherlands), using amplification primers. Sequences were deposited in NCBI and BOLD databases (NCBI acc. numbers MW535774-MW535834; BOLD project CROCU). The BOLD identification tool and NCBI Blast were used for species verification and as a threshold 2% sequence divergence was applied.

Available COI and ITS2 sequences of conspecific and congeneric (where appropriate) mosquito specimens were withdrawn from BOLD and GenBank databases. Sequences were collapsed to haplotypes in FaBox 1.5 online sequence toolbox (Villesen 2007). Multiple sequence alignments were conducted with MAFFT version 7 (Katoh et al. 2019, https://mafft.cbrc.jp/alignment/server/index.html; final alignments available upon request). Statistical parsimony haplotype networks for each species were constructed using TCS 1.21. (Clement et al. 2000) under the 95% parsimony criterion. Neighbor-joining (NJ) trees based on the p-distance model were calculated in MEGA 7.0.25 (Kumar et al. 2016), with 1,000 bootstrap replicates. Maximum likelihood (ML) trees were constructed on PhyML 3.0 web-server (Guindon et al. 2010), with automatic model selection by SMS (Lefort et al. 2017) and aLRT SH-like support (Anisimova and Gascuel 2006). Resulting trees were edited in FigTree v.1.4.3. (http://tree.bio.ed.ac.uk/software/figtree/).

Statistical analysis

Analysis was performed using R (version 4.02) with a significance level of 0.05 and ComEcoPaC (Version 1. http://prf.osu.cz/kbe/dokumenty/sw/ComEcoPaC/ComEcoPaC.xls. Accessed on 24 October 2020). The species diversity was analyzed with the Shannon – Wiener index. Shannon-Wiener's evenness takes values between 0 and 1, where a value closer to zero represents the dominance of one species and less evenness, and a value closer to one represents a more equal abundance of species and complete evenness.

For the similarity of sampling areas, Jaccard's Similarity Index was employed. Using Poisson's regression, the number of mosquitoes based on the predictor of altitude (specifically for *Cx. torrentium* and *Cx. pipiens*) is given by the expressions:

Expected number of *Cx. pipiens* =
Exp^(2.2293531–0.0006178 * altitude

Expected number of *Cx. torrentium* =
0.61650461+0.000572 * altitude

where Exp is the exponential base function e.

RESULTS

A total of 890 specimens (276 females, 128 males, 486 larvae) was morphologically assessed and 16 species were recorded belonging to the genera *Anopheles* (three species), *Aedes* (six species), *Culex* (four species), *Culiseta* (two species), and *Coquillettidia* (one species). The *Culex pipiens* complex was dominant (50% of the sampled specimens), followed by *Culex torrentium* (20.22%). Dominant species were *Culiseta longiareolata*, (8.53%) and *Aedes japonicus* (7.75%). Less represented species were *Culex hortensis* (4.94%), *Anopheles maculipennis* complex (3.48%), *Culiseta annulata* (1.91%), *Aedes punctor* (1.46%), and *Aedes sticticus* (0.89%). For several species, only a few specimens were sampled: *Aedes geniculatus* (five individuals), *An. plumbeus* (four individuals), *Culex territans* and *Ae. communis* (three individuals each), *Coquillettidia richiardii* (two individuals), and *Ae. cantans/annulipes* (only one individual).

In area 1 we collected 301 specimens belonging to ten species (Figure 1A). The diversity index in this sampling area was low, E= 0.5. The eudominant species was the *Cx. pipiens* complex that comprised 65.1% of all recorded individuals followed by *Cx. torrentium* with 18.3%. The *An. maculipennis* complex accounted for 6.6% and *Ae. japonicus* for 4.7% of the collected specimens.

In area 2, we found 12 different species in a total of 346 specimens (Figure 1B). For this area the diversity index was E= 0.65 and the number of species in relation to other areas was the highest. The majority of specimens belonged to the *Cx. pipiens* complex (42.2%); *Cx. torrentium* comprised 24.9%, the invasive species *Ae. japonicus* comprised 15.3%, *Cs. longiarelata* 6.1%, and *Ae. punctor* 3.75% of the collected specimens.

In area 3, ten different species were recorded with a total of 243 specimens (Figure 1C). The value of diversity index for this area was the highest, E= 0.72. The most common species in that area was again *Cx. pipiens* complex with 42.4% of the collected specimens. *Cs. longiareolata* was the second most common species with 22.2%, the third most common species was *Cx. torrentium* which constituted 15.6%, *Cx. hortensis* constituted 7.4%, and *Cs. annulata* comprised 6.2% of all mosquito specimens collected in that area. For the species *Ae. cantans/annulipes,* both species names were retained due to the similarity of the adults within the *annulipes* group (Becker et al. 2010).

Poisson's regression yielded statistically significant results. The Expected Logarithm of a number of species for *Cx. pipiens* decreased by 0.0006178 altitude units. Expected Logarithm of a number of species for *Cx. torrentium* increased by 0.000572 altitude units. The regression indicated that *Cx. pipiens* prefered lower altitudes and *Cx. torrentium* higher altitudes, according to the above values. A higher similarity between species, confirmed with Jaccard's similarity index (Ja), was observed between the areas 1 and 2 and between areas 2 and 3 (Ja=0,5714), and less between areas 1 and 3 (Ja=0,4286).

Figure 2A shows the number of specimens and species sampled at different altitudinal ranges together with the number of sampling sites. From 301 to 500 m, 14.7% of mosquito specimens within the eight different species were collected. At the range of 501 to 700 m, 8.5% mosquito

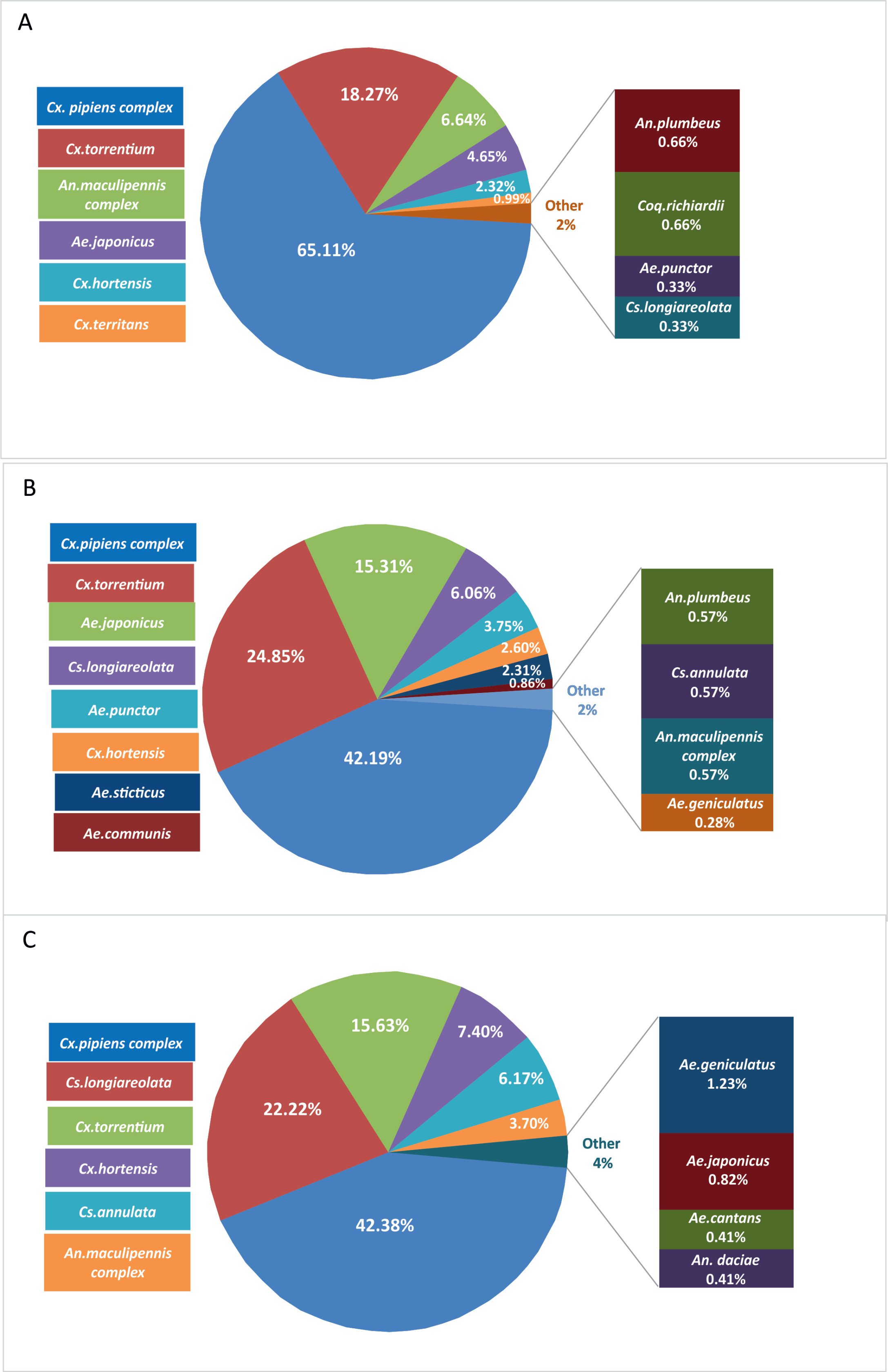
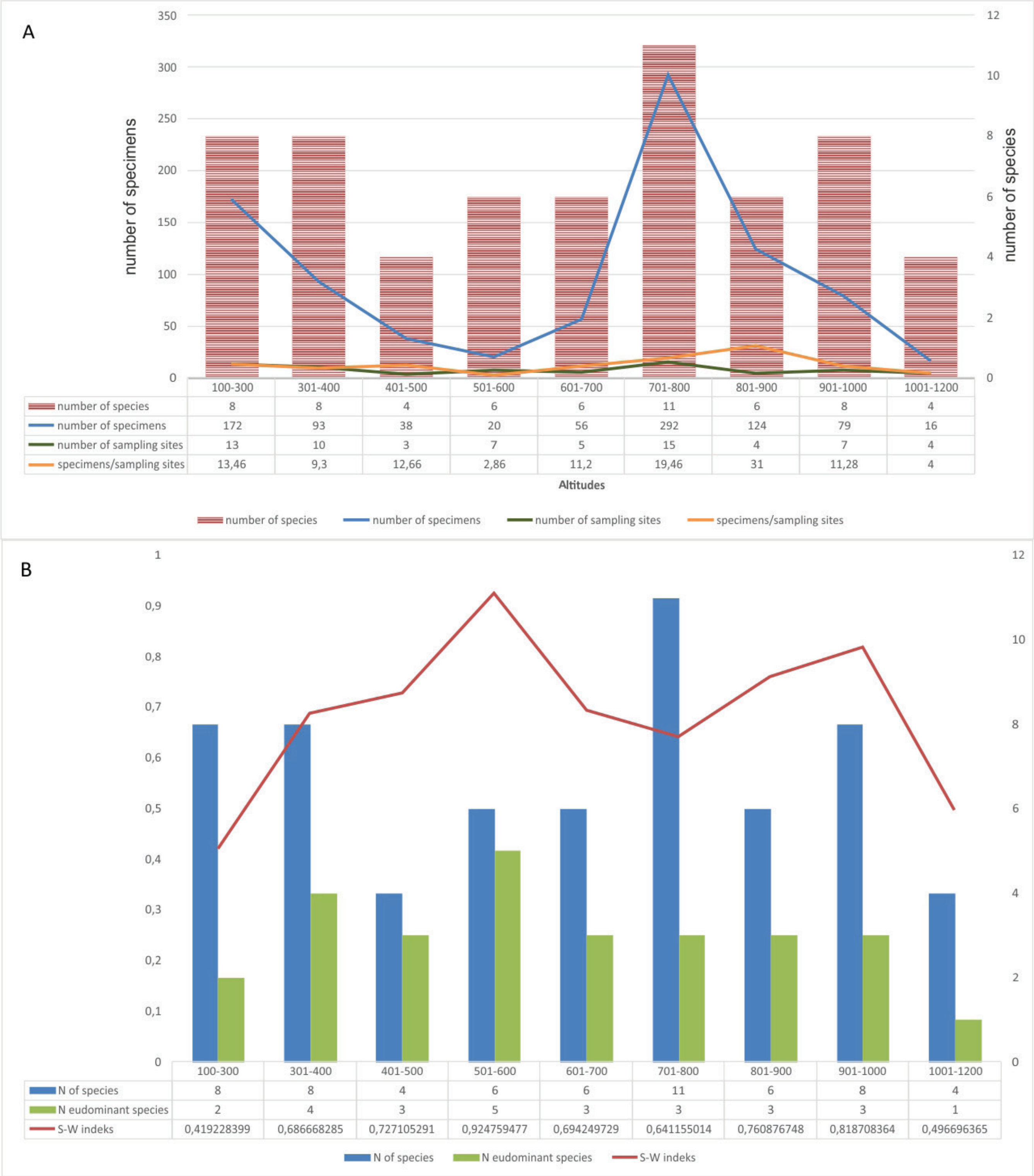


Figure 1. (A) Total of mosquito samples in the area of Papuk; (B) total of mosquito samples in the area of Gorski Kotar; (C) total of mosquito samples in the area of Velebit.



	100-300	301-400	401-500	501-600	601-700	701-800	801-900	901-1000	1001-1200
▨ number of species	8	8	4	6	6	11	6	8	4
— number of specimens	172	93	38	20	56	292	124	79	16
— number of sampling sites	13	10	3	7	5	15	4	7	4
— specimens/sampling sites	13,46	9,3	12,66	2,86	11,2	19,46	31	11,28	4

▨ number of species — number of specimens — number of sampling sites — specimens/sampling sites

	100-300	301-400	401-500	501-600	601-700	701-800	801-900	901-1000	1001-1200
🟦 N of species	8	8	4	6	6	11	6	8	4
🟩 N eudominant species	2	4	3	5	3	3	3	3	1
— S-W indeks	0,419228399	0,686668285	0,727105291	0,924759477	0,694249729	0,641155014	0,760876748	0,818708364	0,496696365

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Figure 2. (A) Number of all sampled specimens, species and sampling sites with respect to altitudinal ranges; (B) Shannon-Wiener's evenness together with the species number and altitudinal range of sampling sites.

specimens within the nine different species were collected. Most specimens (46.7%) and most species (n=11) were recorded at an altitude of 701 to 900 m. At the range of 900 to 1,200 m, 10.7% of mosquito specimens within the nine different species were collected. Members of the *Cx. pipiens* complex were recorded at all altitudinal ranges, with the largest number of specimens (n=179) collected at the range of 701 to 900 m. The highest average number of collected samples was in the range of 801 to 900 m (n = 31) and 701 to 800 (n = 19.46), while the smallest number was in the range of 501 to 600 m (n = 2.86).

Figure 2B shows Shannon-Wiener's evenness together with the species number and altitudinal range of sampling sites. The highest diversity index value (E=0.92) is noted for the altitudinal range from 501 to 600 m, where the number of dominant species (n = 5) relative to the total number of species (n = 6) is large. The lowest diversity index value (E=0.42) is noted for the altitudinal range from 101 to 300 m, where the number of dominant species (n = 2) relative to the total number of species (n = 8) is small. This is also the case within the altitudinal range of 701 to 800 m where the index is lower (E= 0.64), although the total number of species is large (n=11), but the number of dominant species is small (n = 3).

Molecular analysis

We obtained complete COI barcode sequences for 57 mosquito specimens belonging to 15 species in four genera (*Anopheles*, *Aedes*, *Culex* and *Culiseta*). From two individuals of the species *Cq. richiardii,* no positive PCR reaction was obtained, so this species was not barcoded. However, because of its specific morphological characteristics, it is very unlikely to confuse with any other species. Therefore, we consider the identification of these specimens highly reliable. BLAST search and BOLD identification through DNA barcodes confirmed our morphological determinations. Phylogenetic analysis of COI barcodes confirmed the grouping of species in highly supported monophyletic clades with the exception of specimens designated as *Cx. pipiens* complex which are grouped in two clades, furthermore in paraphyly with sibling species *Cx. torrentium* (Figure 3). Average intraspecific genetic distances are generally low (Table 1), with the exception of *Cx. territans* and *Ae. sticticus*. These results are further confirmed by phylogenetic networks constructed for each species. In most species a low number of closely connected haplotypes is observed, with higher genetic variability in *Cx. territans* and *Ae. sticticus* (not shown). At the same time, interspecific genetic distances are much higher (average interspecific distance 0.1275; range 0.0968 - 0.1637), confirming the existence of a barcoding gap.

The 123 specimens, morphologically identified as *Culex pipiens* complex, were analyzed for microsatellite loci markers. *Culex torrentium* was confirmed with 79 specimens (64.2%), 23 specimens (18.7%) were identified as *Cx. pipiens* f. *pipiens* (present in all three areas, the highest altitude was 922 m), three specimens as *Cx. pipiens* f. *molestus* (in areas 1 and 2, the highest altitude was 698 m), and two specimens as their hybrids. Hybrid specimens were collected in area 1 at the altitude of 184 m and in area 2 at the altitude of 776 m.

The ITS2 region sequenced for the specimens morphologically determined as members of *An. maculipennis* complex revealed seven specimens as *An. maculipennis* s. s. and one specimen as *Anopheles daciae* (in the area 3 at 785 m). Members of the *An. maculipennis* complex were found mostly in the range of 301 to 400 m.

DISCUSSION

Recent research on mosquito fauna of neighboring countries mainly deals with vector species and the diseases they transmit. With regard to mountainous areas, some

Table 1. Average genetic distances for COI within the species.

Species	p-distance MIN	p-distance MAX	p-distance AVERAGE
Aedes japonicus	0.00	0.006	0.0039
Anopheles plumbeus	0.00	0.005	0.0006
Culiseta annulata	0.00	0.0015	0.0006
Culiseta longiareolata	0.00	0.0046	0.0008
Culex hortensis	0.00	0.011	0.0076
Culex territans	0.00	0.077	0.0336
Culex pipiens complex	0.00	0.0369	0.0068
Culex torrentium	0.00	0.0187	0.0037
Aedes cantans	0.00	0.016	0.0087
Aedes communis	0.00	0.0091	0.0035
Aedes geniculatus	0.00	0.0304	0.0092
Aedes punctor	0.00	0.008	0.0035
Aedes sticticus	0.00	0.0638	0.0372
Anopheles maculipennis complex	0.00	0.030	0.017

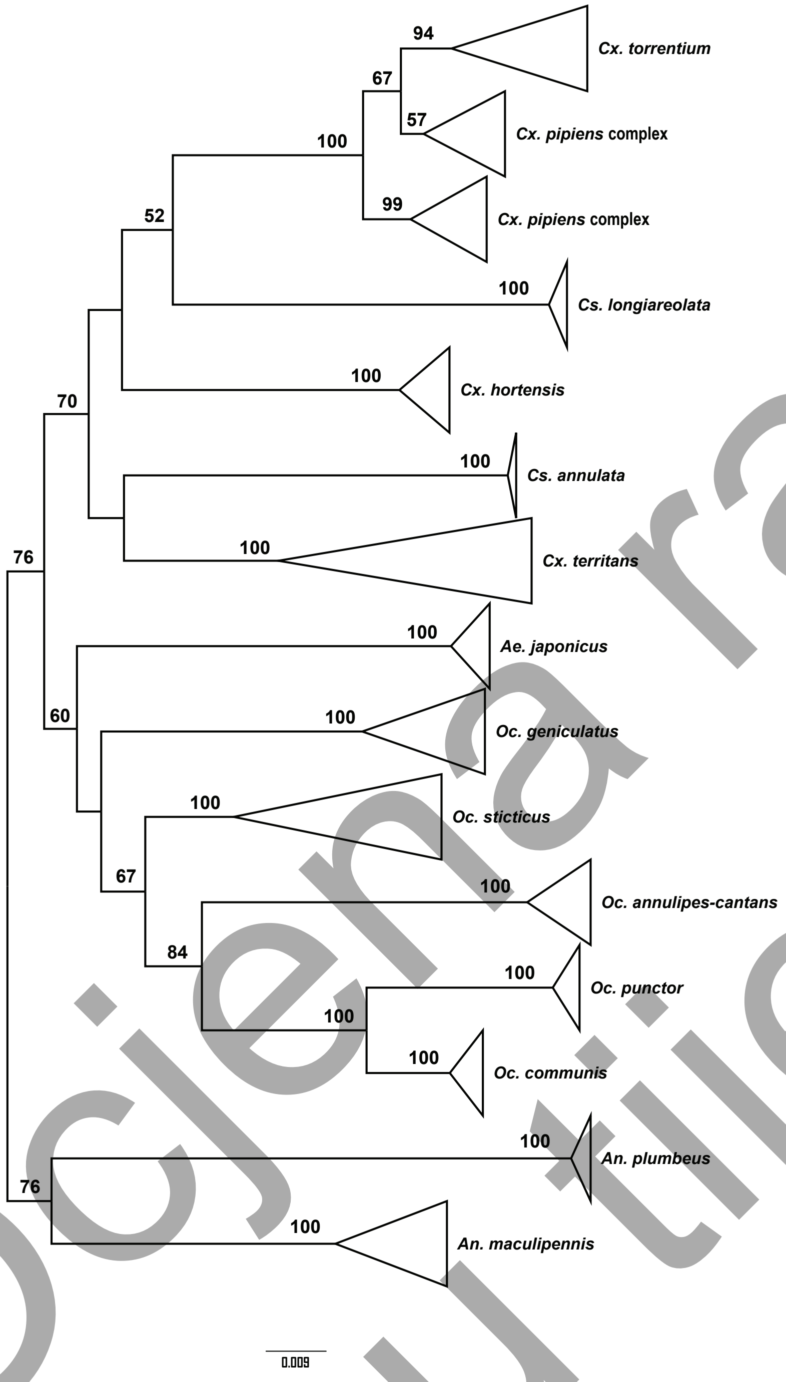

Figure 3. Unrooted maximum likelihood phylogenetic tree based on COI haplotypes of mosquito specimens sampled in this work and conspecific sequences from GenBank. Species clades are collapsed for simplicity; numbers on the nodes denote ML aLRT / NJ bootstrap support (values lower than 0.70 / 50% are not shown).

faunal research was conducted in Serbia and Montenegro during the 1980s (Božičić 1987, Božičić and Morović 1987), however there are no more recent data for the higher parts of neighboring countries.

Our faunal investigation of high-altitude regions in Croatia (Papuk, Gorski Kotar, and Middle Velebit) showed that 16 species of mosquitoes are present in these areas. Mosquitoes were sampled after the rainy season and were found in almost all localities. The mosquito broods were generally small, but despite that, various species were found. The species that were found coincide with the species previously recorded in the Papuk area (Merdić 1995b, Merdić et al. 2018), except for the species *Aedes pulcritarsis*, *Aedes vexans,* and *Aedes cinereus* which were not sampled in our research. On the other hand, we sampled several species which were not recorded in previous investigations: *Cx. hortensis*, *Cx. territans*, *Ae. punctor,* and *Cs. longiareolata*, raising the total number of recorded species of mosquitoes to 17 for these regions.

The distribution of species depends on various biotic and abiotic factors such as temperature, humidity, geographical barriers, and availability of vertebrate hosts. In addition, one of the selective factors is altitude. Recent research conducted in Kosovo has shown that most mosquitoes were sampled at altitudes of 301 to 500 m and that the increase in altitude decreased the number of species and their abundance (Muja-

Bajraktari et al. 2019), which was not the case in our study. In our study, most species and number of samples were recorded in area 2 at altitudes of 601 to 800 m. As area 2 is located mainly at an altitude of 701 to 900, the number of sampling sites in that altitude range was the most numerous. There were also a large number of different habitat types in this area in where populations have been found. Therefore, it was expected that the number of sampled individuals and the number of species would be the highest in this altitudinal range. Although higher altitudes are a natural barrier, this research has shown that mosquitoes can be found in large numbers there as well, with the highest number of sampled specimens/sampling site at the altitudinal range of 701 to 900 m. The Shannon-Wiener's evenness has shown a decrease at these altitudes, similar to research recently conducted in Kosovo (Muja-Bajraktari et al. 2019), which was to be expected. Areas 1 and 2 are very similar in terms of species diversity, which may be explained by the fact that there are similar types of breeding sites for mosquito development.

Members of the *Cx. pipiens* complex represent the most important vectors for various arboviruses including West Nile virus (WNV), which has been present in Croatia since 2012 (Pem-Novosel et al. 2014). We found them at all altitudinal ranges, mostly at 270 to 720 m, which was also the case in the recent study in Kosovo (Muja-Bajraktari et al. 2019). The vicinity of the houses where we sampled and the ability of these mosquitoes to inhabit nearly every kind of water body (Korba et al. 2016) makes the *Cx. pipiens* complex the eudominant species in our research. The *Culex pipiens* complex is subdivided into two morphologically indistinguishable biotypes, *Cx. pipiens* biotype *pipiens* and *Cx. pipiens* biotype *molestus*, but so far in Croatia, these two biotypes have been recorded as the *Cx. pipiens* complex. The only research in which the two biotypes were discerned based on their mode of hibernation, homodynamic or heterodynamic (Vinogradova 2000), was conducted in the areas of Zagreb and Osijek (Merdić and Vujčić-Karlo 2005) and they showed the presence of the biotype *molestus*. Based on molecular determination, our research has confirmed the presence of both *Cx. pipiens* biotypes, as well as their hybrids, in the researched high-altitude areas, although with varying frequencies. A small number of the biotype *molestus* can be explained by their preference for urban habitats (Osorio et al. 2013, Vogels et al. 2016), of which there were few in our study. The importance of identifying hybrids between these two biotypes is that they can represent bridge vectors between infected birds to humans and facilitate the transmission of WNV (Fonseca et al. 2004, Gomes et al. 2013).

The presence of sibling species of *Cx. pipiens* complex and *Cx. torrentium* was noted in the study by Merdić et al. (2018) in the Papuk area. In our study, these two sibling species were distinguished on the basis of differences between male genitalia and on molecular analysis. Our results confirm previous observations that *Cx. torrentium* prefers higher altitudes (from 127 to 933 m), which was also shown by Poisson's regression. At 25 locations, the presence of both species was noted in the same habitats, which has been the case in previous studies as well (Service 1968, Hesson at al. 2014, Merdić et al. 2018).

Among the species found, the invasive mosquito species *Ae. japonicus* has been identified and confirmed by DNA barcoding. Because of its cold tolerance that allows an expanded seasonal activity range in temperate climates (Kaufman and Fonseca 2014) and the fact that the larvae and pupae can tolerate a wide range of water temperatures (Cunze et al. 2016, Reuss et al. 2018), it is not unexpected that it was found at higher altitudes in July and August (up to 1,185 in the area of Middle Velebit). Moreover, in a study conducted by Bevins (2007), *Ae. japonicus* was collected at all sampled elevations (425-1,500 m), as well as in Upper Bavaria, Germany with minimum regional altitudes c. 400 m (Zielke et al. 2016). Considering its ability to adjust, it was noted that *Ae. japonicus* suppresses populations of other mosquito species in many areas of North America and Europe, which directly affects biodiversity (Kampen and Werner 2014). However, the possibility of cohabitation of *Ae. japonicus* with other species has been shown recently (Montarsi et al. 2019). Results of our study confirm the suppressing characteristic of *Ae. japonicus,* since it was predominantly found together with *Culex* species and only in several cases together with some other species. *Ae. japonicus* has the highest abundance at about 701 m while the number of specimens decreases at lower or higher altitudes, which points to the fact that higher altitudes do not represent a barrier to the spread of this species and that, in southeastern Europe, a further spread is anticipated (Janssen et al. 2020).

Molecular characterization of the *An. maculipennis* complex revealed the species *An. maculipennis* s. s., which was found in all three researched areas, and *An. daciae,* which was found only in Middle Velebit. The first record of *An. daciae* for Croatia was in 2014 in Slavonia and Lika (Vignjević 2014, unpublished Ph.D. dissertation). The highest altitude at which it was found was 560 m (Ričice, Lika), together with the species *An. maculipennis* s. s., as was the case in our study as well, but at an altitude of 785 m. In recent research from Kosovo (Muja-Bajraktari et al. 2019), the abundance of the members of the *An. maculipennis* complex was much higher than in our research, but nevertheless a large number of samples were found at the same altitude range (301 - 400 m).

Climate change may lead to an increase in environmental temperatures that would consequently affect the biology and ecology of a number of vectors, including mosquitoes. High altitude areas were previously unsuitable for the spread of mosquito vector populations, but as a result of global warming they could become suitable environments for mosquitoes. As a consequence of global warming, the spread of the *An. maculipennis* complex towards northeastern Europe and northwestern Asia and changes in the composition of the *Anopheles* species significantly affects the deterioration of the epidemiological situation of malaria infection in Eurasia (Novikov and Vaulin 2014).

The COI barcoding fragment proved to be useful for discrimination between mosquito species, with the exception of particular closely related taxa. In our work, generally low intraspecific and high interspecific genetic variability, consistent with a barcoding gap, is observed for most of the species. The exceptions are species of the *Cx. pipiens* complex, *Cx. territans* and *Ae. sticticus,* which bear high levels of intraspecific genetic variability. For the *Cx. pipiens* complex, COI appears to be an unreliable genetic marker in many instances (Shaikevich 2007, Laurito et al. 2013, Shahhosseini et al. 2020). Our results confirm this fact, since in the COI phylogenetic tree, samples of *Cx. pipiens* complex are grouped in two separate clades, furthermore in paraphyly with the sibling species *Cx. torrentium*. However, nuclear microsatellite loci ACE2 and CQ11 successfully discriminated between these taxa, similar to other recent publications (Zittra et al. 2019). Our finding of relatively high intraspecific genetic variability for species *Cx. territans* and *Ae. sticticus* is also in accordance with the results observed in some previous barcoding investigations (Versteirt et al. 2014, Hernández-Triana et al. 2019), suggesting the gradual establishment of deep evolutionary splits in these two species. More thorough analyses by additional molecular markers and more comprehensive sample collections are needed in order to clarify these questions.

On the other hand, for the species pair *Ae. annulipes/cantans,* the COI region does not provide enough information to assign the specimens to particular species. This finding is not unexpected, because it was already shown that within the genus *Aedes* there are several sister-species pairs with very close or overlapping sets of variants of COI gene fragments that could therefore not be discriminated by barcoding (Khrabrova et al. 2013). The authors suggest that morphological determination of the 4th stage larva is a more reliable method for distinguishing between species in genus *Aedes* (Khrabrova et al. 2013).

In conclusion, in this study 16 species were recorded, comprising 32.8% of the total number of mosquito species found so far in Croatia (Merdić et al. 2020). Although we inferred moderate mosquito species diversity for these areas, it is to be expected that future investigations will reveal additional species of mosquitoes in higher altitude regions in Croatia. The results obtained in this work contribute to our understanding of mosquito spreading in higher altitude areas, which is especially important for mosquitoes as vectors of numerous vector-borne diseases.

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CHAPTER 2

Contribution to the mosquito fauna (Diptera, Culicidae) of Lika, central Croatia, with special reference to invasive species

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CONTRIBUTION TO THE MOSQUITO FAUNA (DIPTERA, CULICIDAE) OF LIKA, CENTRAL CROATIA, WITH SPECIAL REFERENCE TO INVASIVE SPECIES

Nataša Bušić, Matej Modrušan, Helena Vilc & Enrih Merdić*

Josip Juraj Strossmayer University of Osijek, Department of biology, Cara Hadrijana 8/A,
31000 Osijek, Croatia

Bušić, N., Modrušan, M., Vilc, H. & Merdić, E.: Contribution to the mosquito fauna (Diptera, Culicidae) of Lika, central Croatia, with special reference to invasive species. Nat. Croat., Vol. 30, No. 1, 231–242, Zagreb, 2021.

This study of mosquitoes in Lika, geographically, geologically and climatically a very specific region, was conducted to gain insight into the composition and relative abundance of the mosquito fauna, as well as to check for the possible presence of invasive species. Sampling took place from July to September 2020. Mosquito larvae were captured from medium and small breeding sites using a 25 cm diameter net and a plastic dipper. Adult specimens were sampled in both urban and rural areas using CO_2 baited CDC traps, CO_2 baited BG Sentinel traps with BG Lure and human landing catch. The study was conducted at 69 sites. We collected 5,126 specimens (100 adults and 5,026 larvae) and demonstrated the presence of 16 mosquito species. CDC traps were almost five times more effective (quantity and quality) than BG Sentinel traps. The most common mosquito taxa caught in Lika were *Culex pipiens* complex (78.36% of all larvae) and *Ae. geniculatus* (30.26% of all adults). Both invasive mosquito species present in Croatia, *Aedes albopictus* and *Ae. japonicus*, were recorded, with *Ae. albopictus* being detected in Lika for the first time. *Aedes japonicus* was widespread, recorded in 23 localities. According to molecular analysis, only *An. maculipennis* s.s. was confirmed in the *An. maculipennis* complex. Within the *Cx. pipiens* complex, both *Cx. pipiens* biotypes, *pipiens* and *molestus* were confirmed together with one single hybrid specimen. Analysis of mosquito occurrence with regard to altitude shows that most samples and species were collected between 601 and 700 m a.s.l., although the Shannon evenness index and Hill's index show the highest value in the range of 901 to 1140 m a.s.l. In this systematic study on the mosquito fauna in Lika, a significant species diversity, including invasive species, was found.

Key words: mosquitoes, fauna, invasive species, altitude dispersal, Lika

Bušić, N., Modrušan, M., Vilc, H. & Merdić, E.: Prilog poznavanju komaraca (Diptera, Culicidae) Like, središnja Hrvatska, s posebnim osvrtom na prisutnost invazivnih vrsta. Nat. Croat., Vol. 30, No. 1, 231–242, Zagreb, 2021.

Istraživanje komaraca u Lici, geografski, geološki i klimatski vrlo specifičnoj regiji, provedeno je kako bi se stekao uvid u faunu i brojnost komaraca, kao i da bi se utvrdila moguća prisutnost invazivnih vrsta. Uzorkovanje se odvijalo od srpnja do rujna 2020. godine. Ličinke komaraca uzorkovane su u srednjim i malim leglima mrežicom promjera 25 cm i plastičnim posudicama. Uzorkovanje odraslih komaraca u urbanim i ruralnim područjima obavljeno je koristeći CDC klopke uz suhi led kao atraktant, BG Sentinel klopke uz BG Lure i suhi led kao atraktant i metodom čovjek-aspirator. Istraživanje je provedeno na 69 postaja. Uzorkovano je 5126 jedinki (100 odraslih i 5026 ličinki) i utvrđena prisutnost 16 vrsta komaraca. CDC klopke bile su gotovo pet puta kvantitativno učinkovitije od BG Sentinel klopki. Najrasprostranjenija i najbrojnija vrsta komaraca uzorkovana u Lici bila

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[*] corresponding author: enrih@biologija.unios.hr

je *Culex pipiens* kompleks (78,36% svih ličinki) i *Ae. geniculatus* (30,26% svih odraslih). Zabilježene su obje invazivne vrste komaraca prisutne u Hrvatskoj, *Aedes albopictus* i *Ae. japonicus*, s tim da je *Ae. albopictus* prvi put zabilježen u Lici. *Ae. japonicus* je široko rasprostranjen, zabilježen na 23 postaje. Prema molekularnoj analizi, unutar kompleksa *An. maculipennis* potvrđena je prisutnost samo *An. maculipennis* s. s. Unutar kompleksa *Cx. pipiens*, potvrđena su oba biotipa, *Cx. pipiens pipiens* i *Cx. pipiens molestus* te jedna hibridna jedinka. Analiza pojave komaraca s obzirom na nadmorsku visinu pokazuje da je većina uzoraka i vrsta prikupljena na nadmorskoj visini od 601 do 700 m, iako Shannonov indeks ravnomjernosti i Hillov indeks pokazuju najveću vrijednost na nadmorskoj visini 901 do 1140 m. Ovim sustavnim istraživanjem faune komaraca u Lici utvrđena je značajna raznolikost vrsta, uključujući i invazivne vrste.

Ključne riječi: komarci, fauna, invazivne vrste, rasprostranjenost na nadmorskim visinama, Lika

INTRODUCTION

Lika is a geographical region in southwestern Croatia. It forms a plateau between Velebit Mountain to the west and south, Lička Plješivica Mountain to the east and Kapela Mountain to the northwest. The northern border is rather vague because the Ogulin-Plašćanska valley forms a transitional area between Lika and Gorski Kotar. Lika is specific for its karst relief, formed of permeable rocks mainly composed of limestone and dolomite. The climate is continental to mountainous, resultant upon Velebit being a barrier.

Because of the permeable substrate (karstic), water does not stay on the surface for long, and there are no significant stagnant water bodies. Nevertheless, due to the high amount of precipitation, there are many potential habitats for the aquatic development of mosquitoes, since water may accumulate in plenty of man-made constructions and containers such as canals, pools, rainwater barrels, buckets, machinery, tyres, etc.

Information on the geographical distribution of mosquitoes is very important for mosquito control and the management of mosquito-borne diseases (Gimnig *et al.*, 2005; Palaniyandi, 2014). The abundance of mosquitoes depends primarily on biotic factors, but abiotic factors related to climate and landscape also play a significant role (Honghoh *et al.*, 2012). To better understand all changes occurring globally, data on the occurrence of mosquito species are needed, especially of invasive species. Although mosquitoes are vectors of pathogens of various diseases, not a single case of vector-borne disease has recently been reported from the Lika area. This could be a reason why this area has not been of particular interest to researchers.

Mosquitoes are well studied in many areas of Croatia (Merdić *et al.*, 2020a). So far, 52 mosquito species have been recorded in Croatia (Merdić *et al.*, 2020b), two of which are the invasive species *Aedes albopictus* and *Aedes japonicus*. Since their first detection (Klobučar *et al.*, 2006, 2014), these two species have spread to most parts of Croatia (Capak *et al.*, 2017; Janssen *et al.*, 2020). Due to the small population and relatively low numbers, the mosquitoes of Lika have been discussed in only a few papers (Adamović & Paulus, 1985.; Vignjević, 2014.; Merdić *et al.*, 2018; Janssen *et al.*, 2020; Bušić *et al.*, 2021), in which 11 species were recorded.

Accurate identification of mosquitoes is critical to establish effective mosquito control programmes and strategies. Identification is commonly based on morphological determination of females and fourth-stage larvae, but this is not possible if parts of the mosquito needed for determination are damaged or missing (Hebert *et al.*, 2003). Moreover, mosquitoes very often occur as complexes of species, the members of which are difficult to distinguish morphologically, or the differences required for determination are limited to a certain sex or life stage of a particular species (Khrabrova *et al.*, 2013). Therefore, morphological determination must often be supplemented by molecular determination.

Despite the low health-related impact of mosquitoes in Lika and the relatively low abundance, mosquito research in this area may be interesting from the point of view of biodiversity in a karst area and the spread of invasive species.

STUDY AREA

According to the climate classification of Koppen, the area of Lika belongs to the climate class “Cfsbx” (C – temperate rainy climate, fs – no dry periods and the highest monthly precipitation in the cold part of the year, b – the warmest months of the year have an average temperature of less than 22°C, x – two maxima in the annual course of precipitation in autumn and winter/spring), while the mountain peaks (above 1200 m a.s.l.) belong to the class “Dfsbx” (D – snow-forest climate). Due to the strong orographic indentation of the Lika area, mean annual air temperatures range from 5°C to 9°C (in winter (January) from –4°C to 0°C and in summer (July) from 15°C to 20°C). In terms of the annual pattern of monthly precipitation, the Lika region belongs to the maritime type. The average precipitation ranges from 1200 to 1800 mm, with more precipitation in the cold part of the year. The maximum occurs in November and the minimum in July. A snow cover of at least 30 cm remains on the ground on the Lika plateau for 18 days in January.

MATERIAL AND METHODS

The sampling took place from July to September 2020 in the Lika region. In Fig. 1, all sites covered by this survey are presented according to sampling method: CDC traps (ten sites), BG-Sentinel traps (five sites), larval sampling (53 sites) and human landing catch (one site). All sampled sites were georeferenced using the mobile application GPS Essentials. Altitude of sampling sites ranged from 413 to 1139 m.

Mosquito collection

During this study, both larval and adult mosquitoes were collected. Larvae were sampled from medium-sized breeding sites (1 – 100 m^2) with a 25 cm diameter net and from smaller water bodies ($\leq$ 1 m^2), such as used car tyres, barrels, buckets, vases, cans, tubs, and tree holes with plastic dippers. Adult specimens were caught in both urban and forested areas using a variety of methods: CDC traps baited with CO_2, BG-Sentinel traps (BGS) baited with CO_2 and BG Lure, and human landing catch. CDC and BGS traps were operated three times during the season (Tab. 1). Traps were set at dusk, collected in the morning and run for approximately 12 hours. The areas where the CDC traps were set were carefully selected for microclimatic conditions suitable for mosquitoes, such as shaded places with vegetation. BGS traps were set attyre repair shops . Human landing catch was performed only once at dusk during the high activity period of mosquitoes. The sampling methods are described in detail in Merdić *et al.* (2020a).

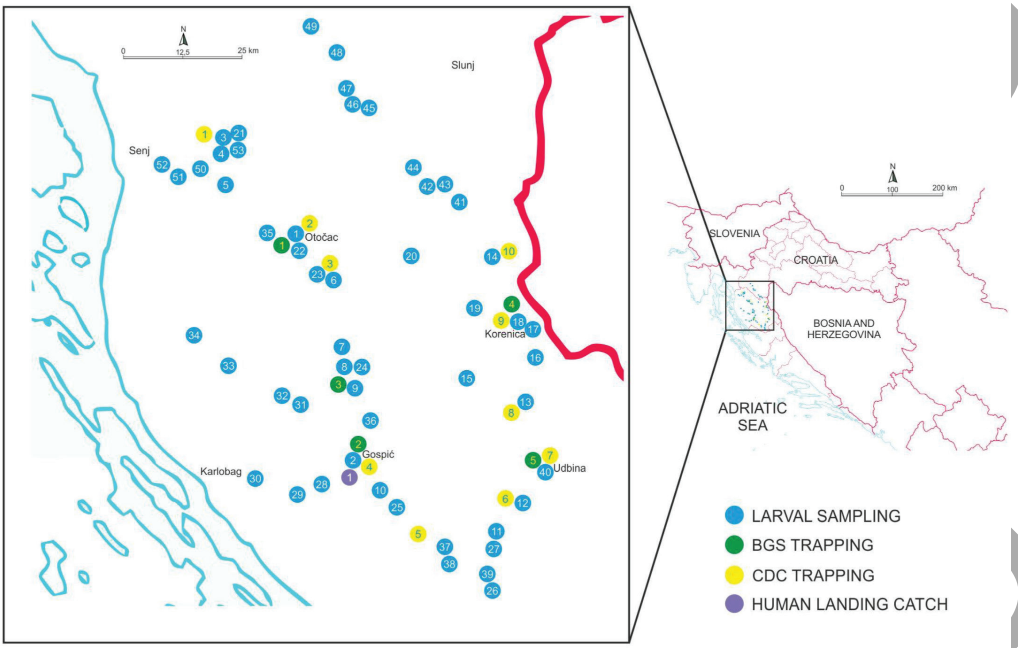

Fig. 1. Mosquito sampling sites in Lika

Legend: Larval sampling: 1. Otočac, 2. Gospić, 3. Brinje, 4. Brinje 2, 5 jezero Gušić, 6 Ličko Lešće, 7. Ličko Lešće, 8. Kvarte, 9. Perušić, 10. Bilaj, 11. Gornja ploča, 12. Kurjak, 13. Pećane, 14. Jezerce, 15. Bunić, 16. Bijelo Polje, 17. Korenica, 18. Korenica, 19. Humoljac, 20. Donji Babin Potok, 21. Brinje, 22. Otočac, 23. Ličko Lešće, 24. Kvarte, 25. Lički Ribnik, 26. Sveti Rok, 27. Lovinac, 28. Trnovačko Novoselo, 29. Brušane, 30. Baške Oštarije, 31. Vranovine, 32. Aleksinica, 33. Pazariški Bakovac, 34. Sj. Velebit, 35. Otočac, 36. Lički Osik, 37. Papuča, 38. Raduč, 39. Sv Rok (ili Jurjevići?), 40. Udbina, 41. Poljanak, 42. Kuselj, 43. Kuselj, 44. Saborsko, 45. Lapat, 46. Lapat, 47. Plaški, 48. Vojnovac, 49. Josipdol, 50. Žuta Lokva, 51. Melnice, 52. Vratnik, 53. Brinje

BG Sentinel trapping: 1. Otočac, 2. Perušić, 3. Gospić, 4. Udbina, 5. Korenica

CDC trapping: 1. Brinje, 2. Otočac 3. Ličko Lešće, 4. Gospić, 5. Medak, 6. Kurjak, 7. Udbina 8. Pećane, 9. Korenica, 10. Jezerce

Human landing catch: 1. Gospić

All sampled adult mosquitoes were killed by freezing at temperatures below 0 °C or with cigarette smoke and mounted on entomological pins. The sampled larvae were preserved in 96% alcohol for molecular analysis.

Mosquito identification

All mosquitoes were morphologically identified according to Gutshevich *et al.* (1974) and Becker *et al.* (2010). Cryptic species within the *Anopheles maculipennis* complex were identified using molecular methods based on polymerase chain reaction of ribosomal DNA according to Di Luca *et al.* (2014). Following Smith & Fonseca (2004) and Bahnk & Fonseca (2006), the *Culex pipiens* complex was analysed for occurrence of *Cx. torrentium* and the *pipiens/molestus* biotypes of *Cx. pipiens* s.s. Three specimens of that complex were taken from each locality for molecular analysis, if there was more than one. All mounted mosquitoes are kept in the entomological collection of the Department of Biology, Josip Juraj Strossmayer University in Osijek.

Tab. 1. Total number of individuals collected per locality by different methods during three samplings

date	8–10 July	22–25 July	2–5 Sept.	
locality	number of individuals			method
Brinje	0	0	1	CDC trap*
Otočac	1	2	0	CDC trap
Ličko Lešće	0	4	1	CDC trap
Medak	8	0	2	CDC trap
Gospić	1	19	6	CDC trap
Pećane	1	2	0	CDC trap
Korenica	1	2	0	CDC trap
Jezerce	1	8	11	CDC trap
Kurjak	0	0	1	CDC trap
Udbina	2	0	2	CDC trap
Gospić	-	-	9	HLC**
Otočac	1	0	4	BGS trap***
Gospić	0	0	0	BGS trap
Perušić	4	1	5	BGS trap
Korenica	0	0	0	BGS trap
Udbina	0	0	0	BGS trap

***CDC –** CDC trap baited with CO_2
****BGS –** BG-Sentinel trap baited with CO_2 and BG lure
*****HLC –** Human lending catch

Statistical analysis

The analysis was performed using ComEcoPaC (version 1, Drozd, 2010). Species diversity was analysed using the Shannon evenness index (E) and Hill's index (N2). The Shannon evenness index (E=H'/Hmax) takes values between 0 and 1, where a value closer to '0' represents lower evenness (the dominance of one species) and a value closer to '1' represents full evenness (an even abundance of species). The value of t Hill's index (inverse Simpson index) starts with 1 as the lowest possible value. The higher the value of this index, the greater the diversity. To compare the similarity of the samples, Jaccard's similarity index (Ja) was used.

RESULTS

The total number of mosquitoes sampled in the study was 5,126. One hundred of these were caught as adults, using CDC traps (76 specimens), BGS traps (15 specimens) and human landing catch (9 specimens). All other individuals were collected from different water bodies at the larval stage. A total of 16 species within 5 genera were recorded (*Culex* – 4 species; *Aedes* – 6 species; *Anopheles* – 3 species; *Culiseta* – 2 species; *Coquillettidia* – 1 species). The collected taxa are: *Cx. pipiens* s.s. (biotype *pipiens* and biotype *molestus*), *Cx. torrentium*, *Cx. hortensis*, *Cx. territans*, *Ae. japonicus*, *Ae. albopictus*, *Ae. vexans*, *Ae. geniculatus*, *Ae. sticticus*, *Ae. cantans*, *An. maculipennis* s.s., *An. claviger*, *An. plumbeus*, *Cs. annulata*, *Cs. longiareolata* and *Cq. richiardii*.

In all three CDC trap samplings, the number of mosquitoes ranged from 0 to 19 per site. The highest number of mosquitoes was caught in July. Two localities stand out for the large number of mosquitoes: Gospić with 26 and Jezerce with 20 mosquitoes. At other localities, significantly fewer or no mosquitoes were caught (Tab. 1). Eudominant species were *Ae. geniculatus* (30.26%), collected at localities 4, 5, 7 and 10 (below, localities are marked with numbers according to the map in Fig. 1) and *Ae. sticticus* (27.63%), caught at localities 2, 3, 4, 5 and 8. The dominant species were *Ae. vexans* (14.47%), sampled at localities 2, 3, 4, 6 and 10 and *An. plumbeus* (10.52%), sampled at localities 4 and 10. Other species accounted for 17.10% of the mosquitoes collected. Among them, members of the *Cx. pipiens* complex were sampled at localities 1, 7 and 9, members of the *An. maculipennis* complex at localities 3, 5 and 8, two individuals of *Cs. longiareolata* at locality 9 and one individual of *Cq. richiardii* and *Oc. cantans*, each, at locality 4, and one individual of *Cs. annulata* at locality 10.

Significantly fewer mosquitoes were caught with BGS traps, almost five times fewer than with CDC traps. BGS traps also captured a lower number of species (Tab. 1). In these traps, the *Cx. pipiens* complex was eudominant with a proportion of 86.66% sampled at localities 1 and 3, further individuals were *Ae. geniculatus* sampled at location 1. Using human landing catch in Gospić (Fig. 1, locality 1 – purple dot), seven species were recorded: *An. plumbeus*, *Ae. japonicus*, *Ae. vexans*, *Ae. geniculatus*, *Ae. sticticus*, *Ae. cantans* and *Cq. richiardii*.

Most mosquito samples were collected as larvae in urban (backyards) and rural areas. The largest number of mosquitoes was caught in late July. Overall, the largest proportion belongs to the *Cx. pipiens* complex, which was eudominant here with a proportion of 78.36%, and breeding sites of this species were also the most numerous. Dominant species were the invasive *Ae. japonicus* (9.74%) and *Cs. longiareolata* (5.73%). All other species together accounted for 5.15% of the larvae collected.

The overview of the recorded species by localities is as follows: species of the *An. maculipennis* complex at localities 6, 12, 24, 25, 36, 47; *An. claviger* at localities 6, 23 ,25; *An. plumbeus* at localities 18, 34, 48, 52; *Ae. japonicus* at localities 1, 4, 6, 7, 9, 14, 22, 35, 37, 38, 40–45, 47–52; *Ae. albopictus* at locality 48, *Ae. geniculatus* at localities 22, 34. *Cx. torrentium* at localities 24–26, 28, 30, 34, 37, 40, 41, 43, 45 and 52; *Cx. hortensis* at localities 9, 29, 30, 42, 46 and 52; *Cx. territans* at localities 9, 23, 25, 29 and 4, *Cs. longiareolata* at localities 2, 4, 6, 9, 12, 13, 16, 22, 23, 27, 28, 33, 39, 40, 43, 44, 49, 51 and 52; *Cs. annulata* at locality 13. The most numerous *Cx. pipiens* complex was sampled at all localities except 24–26, 28, 30, 34, 37, 40, 41, 43, 45, and 52 (details below).

Within the *An. maculipennis* complex, only *An. maculipennis* s.s. individuals were confirmed, all of them occurring in the altitudinal range from 413 to 746 m a.s.l. (as adults at localities 3, 5, 8 and as larvae at localities 6, 23, 25). Within the *Cx. pipiens* complex, both biotypes, *pipiens* (n = 22) and *molestus* (n=1), were identified together with one hybrid individual. *Cx. pipiens* biotype *pipiens* was detected at elevations from 401 to 500 m a.s.l. with eight larvae (sites 6, 7, 23, 46), from 501 to 600 m a.s.l. with four larvae (sites 10, 21,36), and from 601 to 700 m a.s.l. with nine larvae (sites 11, 13, 16, 31, 32, 38) and at 783 m a.s.l. with one larva (site 19). The single *Cx. pipiens* biotype *molestus* larva was detected at an altitude of 758 m a.s.l. (site 39) and the hybrid larva at an altitude of 613 m a.s.l. (site 20).

Both invasive mosquito species present in Croatia, *Ae. albopictus* and *Ae. japonicus*, were recorded in this study, *Ae. albopictus* for the first time in Lika. The only individual was found in the village of Vojnovac (Fig. 2), in a used tyre at an altitude of 432 m a.s.l. By contrast, *Ae. japonicus* was recorded with numerous specimens at an altitudinal range from 493 to 806 m a.s.l. Its numbers were highest in the northern part of Lika, along the border with Gorski Kotar (Fig. 2). *Ae. japonicus* was found at 22 out of 52 sites, representing 42.3% of all sites surveyed. The breeding site with the highest numbers of collected specimens (n=77) was located in Žuta lokva (site 50) at an altitude of 533 m a.s.l. Depending on the type of habitat, most of the sampled breeding sites were tyres, followed by barrels, buckets and others. Mostly, individuals of *Ae. japonicus* were found together with species of the genus *Culex*, less frequently with species of the genera *Culiseta, Anopheles* and *Aedes* (Tab. 2). In 20 of 23 localities, *Ae. japonicus* was found together with other species, while it was detected alone at only three localities.

Analysis of elevation shows that the largest numbers of specimens and species (n=14) were collected in an altitude range from 601 to 700 m a.s.l., and the largest number of sampling sites was also located in this altitudinal range (most of the plateau). The fewest specimens were collected in an altitude range of 901 to 1140 m a.s.l., which was where the fewest sampling sites were located (Tab. 3). By contrast, the Shannon evenness index and Hill's index show the highest value in this altitudinal

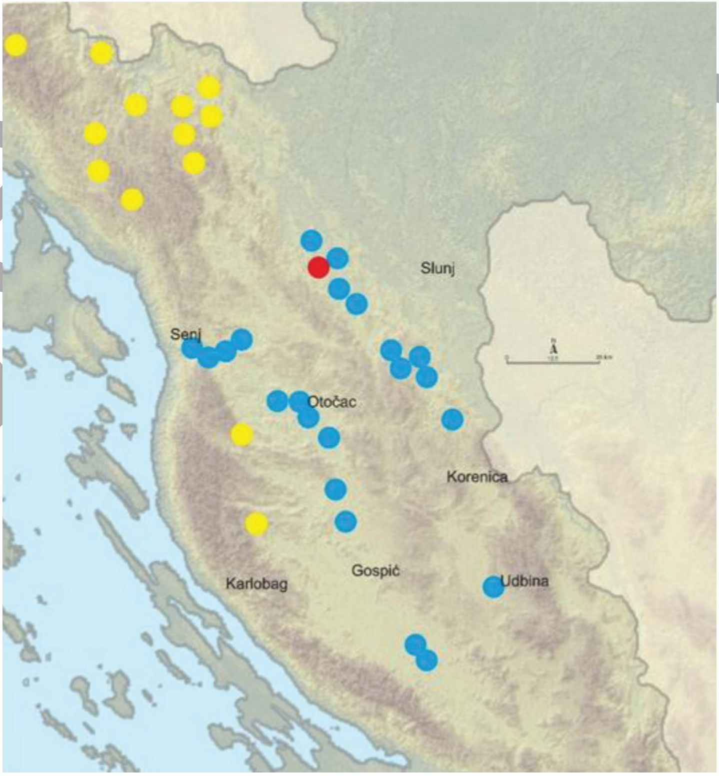

Fig. 2. Distribution of invasive mosquito species of *Ae. albopictus* (red dot) and *Ae. japonicus* (blue dot) in Lika. Yellow dots show the distribution of *Ae. japonicus* according to a previous study (Janssen *et al.*, 2020).

Tab. 2. Number of sites and different habitat types cohabitated by *Aedes japonicus* and other mosquito species

habitat type	barrel	tyre	bathtub	cemetery vase	bucket
species	number of sites				
An. maculipennis s.s.	1				
An. claviger	1				
An. plumbeus		1			
Ae. albopictus		1			
Ae. geniculatus		1			
Cx. pipiens complex	3	7	1	1	4
Cx. torrentium	2	1	1		2
Cx. hortensis	1	1			1
Cx. territans		1			
Cs. longiareolata	5	4	1		

Tab. 3. Total number of specimens and species collected by altitude along with sampling site

species/altitudinal range (m a.s.l.)	401–500	501–600	601–700	701–800	801–900	901–1140	total
An. maculipennis complex	16	33	6	25	0	0	80
An. maculipennis s.s.	9	10	7	5	0	0	31
An. claviger	20	1	0	0	0	0	21
An. plumbeus	1	0	11	4	0	2	18
Ae. japonicus	170	111	65	67	78	0	491
Ae. albopictus	1	0	0	0	0	0	1
Ae. vexans	2	1	6	3	0	0	12
Ae. geniculatus	2	4	6	13	2	2	29
Ae. sticticus	1	9	14	0	0	0	24
Ae. cantans	0	0	2	0	0	0	2
Cx. pipiens complex	474	1547	1537	420	16	12	4006
Cx. torrentium	5	4	13	8	2	3	35
Cx. hortensis	3	0	16	0	9	8	36
Cx. territans	29	15	2	0	0	0	46
Cs. annulata	0	0	1	1	0	0	2
Cs. longiareolata	13	22	190	16	35	14	290
Cq. richiardii	0	0	2	0	0	0	2
number of sampling sites	16	14	23	9	4	3	69
number of specimens	746	1757	1878	562	142	41	5126
number of species	13	10	15	10	6	6	16

range (Fig. 3), where the number of eudominant species was three, while the total number of species was six. The lowest values of the diversity index were obtained in an altitudinal range from 501 to 600 m a.s.l. Accordingly the number of eudominant species was equal to one, and the total number of species was ten. With altitude increasing, the diversity indices also increased, i.e. the dominance of a single species

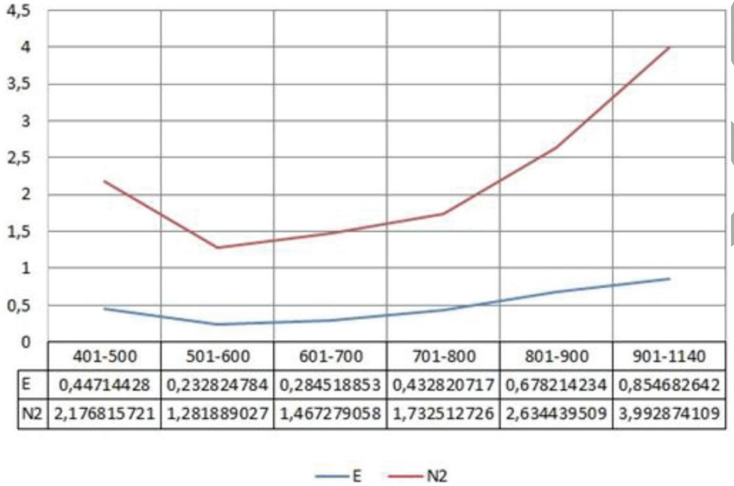

Fig. 3. Shannon evenness index (E-blue line) and Hill's index (N2-red line) as a function of altitude

decreased. The only exception was at heights from 401 to 500 m a.s.l. (Fig. 3). According to Jaccard's similarity index, the highest similarity between localities could be found in the altitudinal ranges from 401 to 500 and 501 to 600 m a.s.l. (Ja = 0.7692), and in the altitude ranges from 801 to 900 and 901 to 1140 m a.s.l. (Ja= 0.7142). The least similarity was found in the elevation range of 501 to 600 and 901 to 1140 m a.s.l. (Ja = 0.3333).

DISCUSSION

Due to the geomorphological and hydrological characteristics, the abundance of mosquitoes in Lika is low. Only a very small number of adult mosquitoes were sampled as compared to larvae (100 out of 5,126 individuals). The larvae were found generally in small to medium sized breeding sites. In addition, these breeding sites were usually located near houses and of were of an artificial nature: barrels, buckets, old tyres dumped in yards, plus gutters and various kinds of debris that retained water and became suitable habitats for mosquito development. In such breeding sites, the house mosquito *Cx. pipiens* s. l. was primarily sampled, in addition to other species that tolerate extremely cold winters, low water levels and higher elevations.

Sixteen mosquito species were recorded in this study. Another two species (*An. messeae* and *An. daciae*) had been recorded in previous studies (Adamović & Paulus, 1985; Vignjević, 2014; Bušić *et al.*, 2021), making a total of 18 species recorded in Lika. This number represents 34.6% of the species known for the Croatian mosquito fauna. This is not a big ratio for such a large area, but considering the particular characteristics and altitude of the Lika region, the number of species is significant.

The fauna of the subfamily *Anophelinae* had previously been studied in this area, with four species of the genus *Anopheles* recorded: *An. claviger*, *An. maculipennis* s.s., *An. messeae* and *An. daciae* (Adamović & Paulus, 1985; Vignjević, 2014; Bušić *et al.*, 2021). *An. claviger* had been found in an altitudinal range from 401 to 500 m a.s.l. (Adamović & Paulus, 1985), as in our study, with the exception of one individual recorded at an altitude of 564 m a.s.l. It is interesting that larvae of this species were sampled on the margins of the Gacka and Lika rivers, i.e. in slowly flowing water. In our study, the most numerous species within the genus *Anopheles* was *An. maculipennis* s.s. A possible reason for not finding *An. messeae* is that this species prefers larger aquatic habitats, especially floodplains of rivers (Becker *et al.*, 2010), of which there are very few in the Lika region. By contrast, *An. maculipennis* s.s. tends to prefer smaller water bodies, which are more frequently represented in the study area. In addition, the elevation of the study area was above 400 m a.s.l., indicating that the species *An. maculipennis* s.s. is better adapted to higher altitudes than *An. messeae*. These data agree with the data of previous studies from other areas, where *An. messeae* was not found above 200 m a.s.l., in contrast to individuals of *An. maculipennis* s.s. and individuals of *An. daciae* (Kronefeld *et al.*, 2014; Vignjević, 2014; Bušić *et al.*, 2021).

Another species within the subfamily *Anophelinae* not previously recorded in Lika is *An. plumbeus*, which was sampled mainly in tyres together with larvae of the *Cx. pipiens* complex, *Ae. geniculatus* and once with *Ae. albopictus*, as well as in barrels with several other species (*Cx. torrentium, Cx. hortensis, Cs. longiareolata*). It should be emphasized that all habitats were close to the forest, which *An. plumbeus* prefers (Gutsevich et al., 1974; Becker et al., 2010).

So far, eight species (*Cx. pipiens* s.s., *Cx. torrentium*, *Cx. hortensis*, *Ae. japonicus*, *Ae. geniculatus*, *Ae. cantans*, *Cs. annulata, Cs. longiareolata*) have been recorded as belonging to the fauna of the *Culicinae* of Lika (Bušić *et al.*, 2021), which were also recorded in this study, and another four species (*Cx. territans*, *Ae. sticticus*, *Ae. vexans*, *Ae. albopictus*) have been recorded for the first time in Lika in this study.

In the last few years, the invasive Asian tiger mosquito *Ae. albopictus* and the Asian rock pool mosquito *Ae. japonicus* have spread throughout Europe, including Croatia (Caminade *et al.*, 2012; Medlock *et al.*, 2012; Klobučar *et al.*, 2019; Koban *et al.*, 2019; Janssen *et al.*, 2020). *Ae. albopictus* has been detected in all counties of Croatia (Capak *et al.*, 2017), while this is the first finding for the geographical region of Lika. During a study conducted in this area in 2017, not a single individual of *Ae. albopictus* was recorded (Bušić *et al.*, 2021). On the other hand, two individuals of *Ae. japonicus* were detected in this area in the locality of Baške Oštarije and Kuterevo (Janssen *et al.*, 2020), while the present study recorded a notable increase in the area occupied by this species. It can be assumed that *Ae. japonicus* has spread from the area of Gorski Kotar to the area of Lika since 2017, as indicated by the largest number of sampled individuals found directly on the border with Gorski Kotar. As in previous studies (Kaufman & Fonseca, 2014; Cunze *et al.*, 2016; Zielke *et al.*, 2016; Montarsi *et al.*, 2019; Bušić *et al.*, 2021), our results confirm the good adaptation of this species to higher altitudes and colder temperatures as well as the possibility of cohabitation with other species, especially those of the genus *Culex*.

The study area extends mainly at higher altitudes (around 600 m a.s.l.), which could be one of the reasons why we recorded the largest number of localities and species, as well as a large number of different habitats, in these altitudinal range from 601 to 700 m a.s.l. Although altitude is a natural barrier, mosquitoes were also found in a range from 901 to 1140 m a.s.l., as in other recent studies (Muja-Bajraktari *et al.*, 2019; Bušić *et al.*, 2021). The Shannon evenness index and Hill's index showed a high value in the altitudinal range from 901 to 1140 m a.s.l., indicating a more uniform abundance of species.

Since the fauna of Lika has only been sporadically examined so far, this is the first systematic study of the fauna of mosquitoes of Lika. Of course, it should be noted that this study probably did not cover all species inhabiting this area, which gives space for further research. Based on the observed spread of the invasive species *Ae. japonicus* in this area since 2017 and the recording of the invasive species *Ae. albopictus,* it is reasonable to assume that the spread will continue and to suggest that it should be monitored.

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CHAPTER 3

A DNA barcode reference library of Croatian mosquitoes (Diptera: Culicidae): implications for identification and delimitation of species, with notes on the distribution of potential vector species

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RESEARCH **Open Access**

A DNA barcode reference library of Croatian mosquitoes (Diptera: Culicidae): implications for identification and delimitation of species, with notes on the distribution of potential vector species

Nataša Bušić[1*], Ana Klobučar[2], Nediljko Landeka[3], Toni Žitko[4], Goran Vignjević[1], Nataša Turić[1,5], Mirta Sudarić Bogojević[1], Enrih Merdić[1], Mladen Kučinić[6] and Branka Bruvo Mađarić[7*]

Abstract

Background Mosquitoes pose a risk to human health worldwide, and correct species identification and detection of cryptic species are the most important keys for surveillance and control of mosquito vectors. In addition to traditional identification based on morphology, DNA barcoding has recently been widely used as a complementary tool for reliable identification of mosquito species. The main objective of this study was to create a reference DNA barcode library for the Croatian mosquito fauna, which should contribute to more accurate and faster identification of species, including cryptic species, and recognition of relevant vector species.

Methods Sampling was carried out in three biogeographical regions of Croatia over six years (2017–2022). The mosquitoes were morphologically identified; molecular identification was based on the standard barcoding region of the mitochondrial COI gene and the nuclear ITS2 region, the latter to identify species within the *Anopheles maculipennis* complex. The BIN-RESL algorithm assigned the COI sequences to the corresponding BINs (Barcode Index Number clusters) in BOLD, i.e. to putative MOTUs (Molecular Operational Taxonomic Units). The bPTP and ASAP species delimitation methods were applied to the genus datasets in order to verify/confirm the assignment of specimens to specific MOTUs.

Results A total of 405 mosquito specimens belonging to six genera and 30 morphospecies were collected and processed. Species delimitation methods assigned the samples to 31 (BIN-RESL), 30 (bPTP) and 28 (ASAP) MOTUs, with most delimited MOTUs matching the morphological identification. Some species of the genera *Culex*, *Aedes* and *Anopheles* were assigned to the same MOTUs, especially species that are difficult to distinguish morphologically and/or represent species complexes. In total, COI barcode sequences for 34 mosquito species and ITS2 sequences for three species of the genus *Anopheles* were added to the mosquito sequence database for Croatia, including one individual from the Intrudens Group, which represents a new record for the Croatian mosquito fauna.

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*Correspondence:
Nataša Bušić
ngraovac@biologija.unios.hr
Branka Bruvo Mađarić
Branka.Bruvo.Madjaric@irb.hr
Full list of author information is available at the end of the article

Conclusion We present the results of the first comprehensive study combining morphological and molecular identification of most mosquito species present in Croatia, including several invasive and vector species. With the exception of some closely related species, this study confirmed that DNA barcoding based on COI provides a reliable basis for the identification of mosquito species in Croatia.

Keywords Culicidae., Cytochrome c oxidase I (COI)., Internal transcribed spacer 2 (ITS2)., Species identification., Species delimitation., Cryptic species., Species complex., Invasive species.

Background

Mosquitoes are one of the most studied groups of insects in the world, mainly because of their medical and veterinary role as vectors of pathogens causing millions of deaths per year.. Although most data on mosquito-borne diseases mainly refer to tropical and subtropical countries, quite a few cases of such infections have also recently been recorded in Europe [1–4]. Due to increasing trade and travel, invasive mosquito species are introduced and spread very easily [5–7], such as *Aedes albopictus* and *Aedes aegypti,* which are vectors of many viruses that cause various infectious diseases (e.g. dengue, chikungunya, yellow fever, Japanese encephalitis, West Nile and Zika viruses) [8–10]. In Croatia, well established populations of *Aedes albopictus* are present [11], while *Aedes aegypti* has not yet been recorded. In 2010, autochthonous cases of dengue fever caused by the dengue virus were registered for the first time in Croatia [12]. Also, several smaller dengue epidemics were continuously registered in Europe, with a number of autochthonous cases in France, Spain and Italy [13–15]. Although invasive species pose a serious medical problem, native mosquito species such as those of the genus *Culex* should not be ignored. This has been demonstrated by recent cases of West Nile virus (WNV) outbreaks in Romania, Spain, the Netherlands, Italy, Hungary, Germany, and Serbia [3, 16–18]. In Croatia, the first clinical cases of WNV infection were reported in 2012 [19], associated with *Culex pipiens* complex, and possibly some other species [20]. After 2012, WNV infections occurred continuously in Croatia [21]. The main vector of Usutu virus (USUV) infection is *Culex pipiens*, although the virus has also been found in several other species [22, 23]. Recent studies show that USUV has become endemic in northwestern Croatia [24]. Malaria used to be a prevalent disease in Europe, but currently only imported cases are reported; no deaths occurred following autochthonous infections in 2000–2019 [25]. However, numbers of imported malaria cases in Europe have increased, leading to the re-emergence of indigenous cases in Greece, Spain, Italy, and France [26–29]. Within the *Anopheles maculipennis* complex, several species are considered vectors of malaria parasites in Europe: *Anopheles atroparvus, Anopheles labranchiae, Anopheles messeae* and *Anopheles sacharovi* [30, 31].

The correct identification of mosquitoes is an important part of implementing effective vector management strategies. Conventional identification with dichotomous keys is essential, but has many shortcomings and is not always sufficient to identify mosquito specimens. Important features required for accurate morphological identification of mosquitoes often fall off or are damaged during sampling (scales, legs, wings), or these defining differences are only visible at a certain stage of development or are related to sex. In addition, mosquitoes often occur in complexes of closely related species, and morphological identification proved to be insufficient in most of these cases [32].

Molecular identification by DNA barcoding is an accurate method of species identification, independent of the developmental stage and condition of the specimens examined [33–35]. Many studies have confirmed that the standard mitochondrial COI barcoding region is a suitable marker for identification of mosquito species and recognition of cryptic species [36–41]. Nevertheless, for several genera and species complexes (*Culex, Aedes* and *Anopheles*), the use of additional molecular markers is necessary to increase identification accuracy and to distinguish between closely related species, forms and hybrids. For example, ITS2 (nuclear ribosomal internal transciber spacer 2), Ace2 gene and polymorphisms of various microsatellite loci (e.g. CQ11) are now routinely used [42–47]. However, despite major advances in molecular methods, accurate identification requires a multidisciplinary approach to taxonomy that includes morphological, molecular, distributional and ecological data [36, 48].

To date, 52 species of mosquitoes have been recorded in Croatia, two of which are invasive: *Aedes albopictus* and *Aedes japonicus* [11]. Traditionally, the majority of research has been based on morphological identification, but several recently published papers have used molecular methods to confirm the presence of certain species. By these, *Cx. torrentium* was proven for the first time to belong to the mosquito fauna of Croatia in 2018 [49], and the presence of several other species was confirmed for certain regions of Croatia [50, 51]. However, no systematic survey has been conducted to date that would provide an overview of the barcodes of the Croatian mosquito fauna, and there is also very little data for neighbour countries [52, 53]. The importance of such data lies in the potential presence and spread of new invasive species that are potential arbovirus vectors, such as *Aedes koreicus*, which is currently established in surrounding countries but has not yet been recorded in Croatia [54, 55]. Recent DNA barcoding studies in Europe [56–64] established national DNA barcode libraries for several countries, and resulted in new species findings and confirmation of unrecorded species in certain areas, as well as identification of cryptic taxonomic units [65]. Nevertheless, the number of barcode sequences of European mosquitoes with accurate country and species designation is still limited and accounts for less than 10% of all Culicidae records in BOLD (accessed December 15, 2023).

The aim of this study was to create a DNA barcode library for the Croatian mosquito fauna and to gain insight into the genetic diversity and geographical distribution of mosquito species through DNA barcoding. The data obtained will contribute to public databases (BOLD, NCBI GenBank) and help create a platform for easier, faster and more accurate identification of mosquitoes of the Croatian fauna. The results will also serve as a basis for projects on the surveillance of invasive and vector mosquitoes and control of mosquito-borne diseases in the studied region.

Methods

Study area

With its geographical location in Central Europe and the Mediterranean region, Croatia is one of the "hotspots" of European biodiversity. The high level of biodiversity in Croatia is a consequence of the diverse composition of habitats, climatic and hydrological characteristics and the complex geological history of the region.

According to Bertić et al. [66], Croatia was divided into three biogeographic regions for the purpose of this study:the Pannonian-Peripannonian region in the north and east, the central mountainous region in the middle and the Mediterranean region in the south (Fig. 1). From 2017 to 2022, 50 sites were sampled in the Pannonian-Peripannonian region, 38 in the central mountainous region and 52 in the Mediterranean region of Croatia (Fig. 1; Additional file: Table S1).

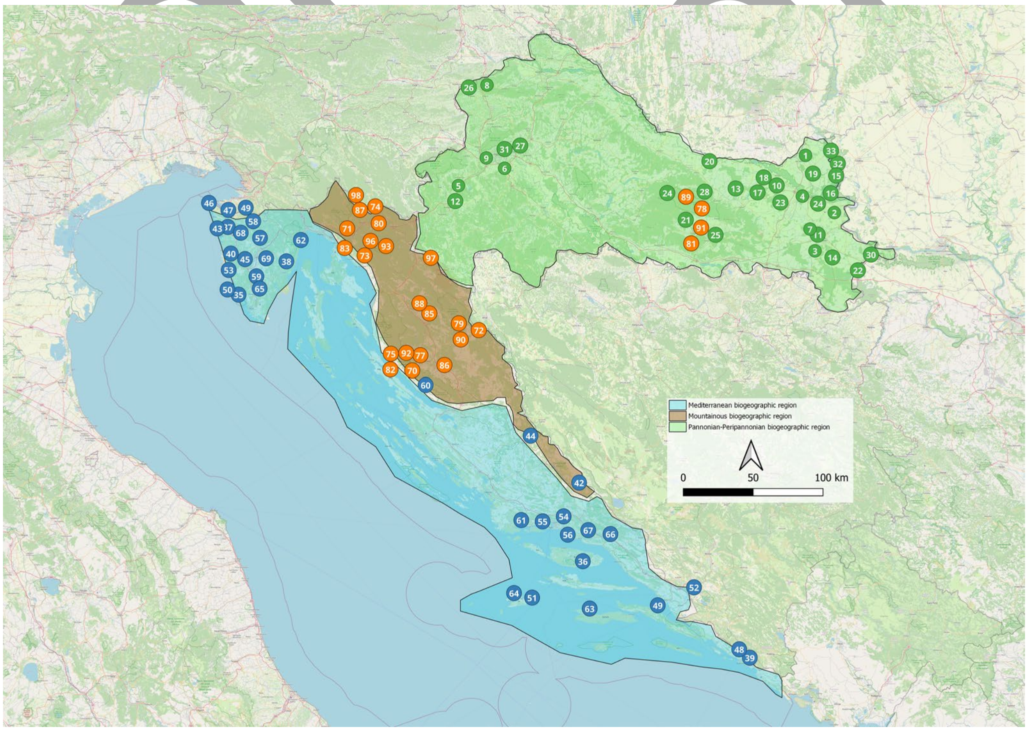

Fig. 1 Sampling localities in three biogeographic regions of Croatia: Pannonian-Peripannonian region—green dots, Mediterranean region—blue dots, and mountainous region—orange dots; high-altitude localities in the Pannonian-Peripannonian region are also marked with orange dots. Some localities may represent multiple sampling sites (details for each sample are available in the public BOLD dataset DS-CROCU2)

Mosquito collection and a priori morphological identification

Adult mosquitoes were caught with CDC traps baited with CO_2, BG-Sentinel traps baited with BG-Lure and CO_2 and human landing catch (HLC) method. Larvae were caught individually using a dipper. The mosquitoes were morphologically identified using the identification keys of Becker et al. [67]. All specimens were stored in 96% ethanol at -20° C. The vouchers of the barcoded specimens are kept at the Josip Juraj Strossmayer University of Osijek, Department of Biology, and at the Andrija Štampar Teaching Institute of Public Health in Zagreb.

DNA extraction and amplification

DNA was extracted from single legs of adult specimens and from entire larvae using the GenEluteTM Mammalian Genomic DNA Miniprep Kit (Sigma, Taufkirchen, Germany). The protocol for rodent tail preparation as provided with the kit was followed with slight modifications (incubation in proteinase K overnight; final DNA elution in 100 µl elution solution).

For all samples, the standard barcoding region of the mitochondrial COI gene [34] was amplified using the universal primers LCO1490 and HCO2198 [68]. For certain species complexes, the COI gene marker does not provide sufficient resolution, so other genomic regions were used. For the identification of species within the *Anopheles maculipennis* complex, the ITS2 region was amplified with the primers 5.8S (forward) and 28S (reverse) [69]. The amplification mixtures and PCR reaction conditions for COI and ITS2 are described in Bušić et al. [50]. The PCR products were enzymatically purified using the ExoI-rSAP system (NEB, Ipswich, MA, USA) according to the manufacturer's protocol and bidirectionally sequenced at Macrogen Inc. (Amsterdam, The Netherlands) using the amplification primers.

Data analysis

The sequences of the COI and ITS2 regions were verified and edited in Geneious 8.1.4. (https://www.geneious.com) and subsequently deposited in the NCBI GenBank and BOLD databases (GenBank accession numbers PP694665-PP694812, BOLD ID numbers in Additional file 1: Table S1; additional information available in public BOLD dataset DS-CROCU2). The percentage identity of the newly obtained COI and ITS2 sequences with the records in the GenBank database was checked with the BLAST tool using the Megablast algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed December 15, 2023). The BOLD identification tool (http://www.boldsystems.org/index.php/IDS_OpenIdEngine; accessed December 15, 2023) was used to compare COI barcode sequences amplified from our samples with publicly available data in the BOLD database. Available published COI sequences of conspecific and congeneric mosquito specimens were selected from the BOLD database of public records (accessed December 15, 2023) and used for subsequent analyses.

COI and ITS2 sequences were analysed in datasets corresponding to specific genera. Multiple sequence alignments were performed with MAFFT version 7 using the "auto" strategy [70] (https://mafft.cbrc.jp/alignment/server/index.html; final alignments in Additional file 2: Dataset S1). Intraspecific and interspecific p-distances were calculated in MEGA 7.0.25 [71]. Maximum likelihood (ML) trees were generated on the PhyML 3.0 web server [72] (http://www.atgc-montpellier.fr/phyml/), with automatic model selection by SMS (Smart Model Selection; model determined by the AIC selection criterion) [73] and aLRT SH-like support [74].

Species delimitation methods (SDMs) bPTP [75] (https://species.h-its.org/ptp/) and ASAP [76] (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) were applied to genus datasets to verify/confirm the assignment of specimens to specific Molecular Operational Taxonomic Units (MOTUs). As input for bPTP, the inferred ML tree was used, while for the ASAP method, the MAFFT alignment was used. In addition, the BIN-RESL algorithm (Barcode Index Number system in BOLD) [77] assigned the sequences to the corresponding BINs in BOLD. The results of the SDMs are presented in a combined ML tree that includes all newly barcoded specimens. The tree was graphically processed in FigTree v.1.4.3. (http://tree.bio.ed.ac.uk/software/figtree/).

Results

In this study, a total of 405 mosquito specimens were collected and processed; 181 specimens in the Pannonian-Peripannonian region, 122 specimens in the Mediterranean region and 102 specimens in the mountainous region of Croatia. Based on morphological identification, a total of 30 species previously recorded in Croatia were sampled, belonging to six genera (*Aedes*—16 species, *Anopheles*—5 species, *Culex*—5 species, *Culiseta*—2 species, *Coquillettidia*—1 species and *Orthopodomyia*—1 species) (Table 1). For some specimens, such as those of the *Aedes annulipes/cantans/excrucians* and *Aedes cinereus/rossicus* groups or some species complexes such as the *Culex pipiens* and *Anopheles maculipennis* complexes, it was not possible to determine morphologically to which species they belonged.

The largest number of species (28) was sampled from the Pannonian-Peripannonian region, followed by the Mediterranean region (19) and the mountainous region (18). Ten species were recorded in all three regions (Additional file 1: Table S1). One individual from the

Table 1 Mean and maximum intraspecific distance (intra-SP) between individuals of the same BIN (species / MOTUs) and distance to nearest neighbour (NN) BIN in BOLD

BIN	Total BIN members	Taxa (this project)	Specimen count (this study)	Taxa included in the BIN (specimen count)	BIN mean intra-SP (%)	BIN max. intra-SP (%)	Distance to NN BIN (%)	NN BIN (taxa)
BOLD: AAA3748	1688	*Aedes punctor*	2	*Aedes punctor* (280); *Ae. hexodontus* (256); *Ae. abserratus* (125); *Ae. aboriginis* (12); *Aedes* sp. (13); *Ae. diantaeus* (9); *Ae. ventrovittis* (4); *Ae. nigripes* (3); *Ae. communis* (3); *Ae. pionips* (4); *Ae. fitchii* (1); *Ae. decticus* (1); *Ae. intrudens* (1); *Ae.* nr. *punctor* (1)	1.68	4.26	1.76	BOLD:AEI5390 (*Aedes* sp. HANKV_BC_Contig1)
BOLD: AAA4751	6020	*Culex pipiens* complex	17	*Culex quinquefasciatus* (3126); *Cx. pipiens* (2002); *Culex* sp. (29); *Cx. torrentium* (358); *Cx.* nr. *pipiens* (38); *Cx. pipiens* s.l. (275);	0.57	5.02	0.98	BOLD:AEW0334 (*Culex pipiens*)
BOLD: AAA5870	4104	*Aedes albopictus*	7	*Aedes albopictus* (4062); *Ae. aegypti* (7); *Ae. malayensis* (2); *Ae. flavopictus* (1); Culicidae sp. (5); *Aedes* sp. (2); *Culiseta annulata* (1)	0.49	3.56	3.88	BOLD:ACH4817 (*Aedes flavopictus*)
BOLD: AAA6148	613	*Aedes communis*	3	*Aedes communis* (338); *Ae. intrudens* (5); *Ae. punctor* (6); *Ae. sticticus* (4); *Ae. dorsalis* (1); *Ae. pullatus* (2)	0.53	3.38	3.45	BOLD:AAB6338 (*Aedes pionips*)
BOLD: AAA7067	5018	*Aedes vexans*	7	*Aedes vexans* (4881); *Ae.* nr. *vexans* (2) *Ae. sticticus* (2); *Ae. intrudens* (1); *Ae. vexans nipponii* (31); *Ae. vexans vexans* (6)	1.22	7.22	3.66	BOLD:ACZ5331 (JOMOS466-15 *Aedes* sp.)
BOLD: AAA9632	515	*Anopheles maculipennis* s.s *Anopheles maculipennis* complex	2 1	*Anopheles maculipennis* (323); *Anopheles* sp. MBI-36 (96); *An. maculipennis* s.s. (92); *An. messeae* (2); *An. maculipennis* s.l. (1); *An. melanoon* (1)	1.50	3.99	1.33	BOLD:ABY8239 (*Anopheles messeae/daciae*)
BOLD: AAB1098	1465	*Aedes annulipes* *Aedes cantans* *Aedes excrucians/cantans* *Aedes cantans/riparius* *Aedes annulipes/cantans*	1 10 1 1 9	*Aedes excrucians* (368); *Ae. cantans* (166); *Ae. annulipes* (95); *Ae. flavescens* (71); *Ae. stimulans* (58); *Ae. fitchii* (42); *Ae. euedes* (7); *Ae. riparius* (3); *Aedes* sp. (11); *Ae. rusticus* (1)	1.22	3.66	1.76%	BOLD:AAD4406 (SSKUA985-15 *Aedes* sp.)
BOLD: AAB2483	237	*Anopheles hyrcanus*	1	*Anopheles hyrcanus* (63); *An. pullus* (136); *An. pseudopictus* (37)	1.58	5.32	2.09	BOLD:AAA5339 (*Anopheles kweiyangensis*)
BOLD: AAB6945	48	*Culex territans*	7	*Culex impudicus* (26); *Cx. territans* (17); *Cx. pipiens* (2)	0.24	1.66	1.12	BOLD:AEE2449 (*Culex territans*)
BOLD: AAB7911	585	*Aedes caspius* *Aedes zammitii*	4 3	*Aedes caspius* (506); *Ae.* nr. *caspius* (23); *Ae. zammitii* (7); *Ae. dorsalis* (5); *Ae. geniculatus* (1); *Aedes* sp. (3); *Culex perexiguus* (1); *Anopheles atroparvus* (2); *Ae. detritus* (1)	1.15	4.14	1.13	BOLD:ACE6286 (*Aedes dorsalis*)

Table 1 (continued)

BIN	Total BIN members	Taxa (this project)	Specimen count (this study)	Taxa included in the BIN (specimen count)	BIN mean intra-SP (%)	BIN max. intra-SP (%)	Distance to NN BIN (%)	NN BIN (taxa)
BOLD: AAC5210	730	*Aedes japonicus*	11	*Aedes japonicus* (726); *Ae. bhutanensis* (2); *Ae. sierrensis* (1); *Ae. japonicus japonicus* (157)	0.65	3.92	7.56	BOLD:ACB6413 (*Aedes koreicus*)
BOLD: AAD6954	330	*Culiseta annulata*	4	*Culiseta annulata* (322); *Culiseta alaskaensis* (1); *Culiseta subochrea* (4)	0.20	2.08	2.56	BOLD:AAV9075 (*Culiseta subochrea*)
BOLD: AAE3979	44	*Anopheles claviger*	1	*Anopheles claviger* (6)	1.20	3.15	1.82	BOLD:AAM4220 (*Anopheles claviger*)
BOLD: AAF2904	318	*Aedes intrudens/diantaeus/pullatus*	1	*Aedes intrudens* (154); *Ae. diantaeus* (133); *Aedes* sp. (1); *Ae. communis* (1); *Ae. aurifer* (1); *Ae. pullatus* (1)	1.36	3.37	3.37	BOLD:AAU0369 (*Aedes aurifer*)
BOLD: AAI5767	72	*Culex hortensis*	11	*Culex hortensis* (70); *Cx. pipiens* (2)	0.84	1.96	6.40	BOLD:AAZ3152 (*Culex adairi*)
BOLD: AAJ7317	424	*Culex modestus*	2	*Culex modestus* (419); *Culex* sp. (3); *Anopheles* nr. *algeriensis* (1); *Cx. pipiens* (1)	1.12	3.55	1.41	BOLD:AEL0567 (*Culex modestus*)
BOLD: AAM2826	155	*Aedes detritus*	3	*Aedes detritus* (142); *Ae. pulcritarsis* (2); *Aedes* sp. (1); *Anopheles atroparvus* (1); *Culex torrentium* (1)	1.13	3.32	5.57	BOLD:AAC9531 (*Aedes* CNKOS142-14)
BOLD: AAM4220	115	*Anopheles claviger*	1	*Anopheles claviger* (87); *An. claviger* s.l. (25); *Coquillettidia richiardii* (2)	1.00	3.56	1.82	BOLD:AAE3979 (*Anopheles claviger*)
BOLD: AAM5033	59	*Aedes rusticus*	3	*Aedes rusticus* (56); *Ae. cantans* (1)	0.33	1.78	3.56	BOLD:AAD4355 (*Aedes provocans*)
BOLD: AAN1645	84	*Aedes pulcritarsis*	1	*Aedes pulcritarsis* (72);	1.10	2.89	5.14	BOLD:AAB7911 (*Aedes caspius*)
BOLD: AAN3326	92	*Anopheles plumbeus*	6	*Anopheles plumbeus* (90); *An. claviger* s.l. (1); *An. claviger* (1)	0.11	1.19	9.89	BOLD:AEA7027 (*Anopheles veruslanei*)
BOLD: AAP0901	314	*Culiseta longiareolata*	10	*Culiseta longiareolata* (302); *Cx. modestus* (1)	0.27	1.85	2.56	BOLD:AEG1924 (*Culiseta longiareolata*)
BOLD: AAP8897	98	*Aedes cinereus*	8	*Aedes cinereus* (78); *Aedes* sp. (8). *Ae. rossicus* (6); *Ae.* nr. *cinereus* (3)	0.65	3.16	1.43	BOLD:AAP8896 (*Aedes cinereus*)
		Aedes rossicus	1					
		Aedes rossicus/cinereus	2					
BOLD: AAR3271	82	*Anopheles algeriensis*	1	*Anopheles algeriensis* (68)	1.45	3.43	4.23	BOLD:ACR6429 (*Anopheles* sp. nr *algeriensis* MBI2015)
BOLD: AAS0072	180	*Coquillettidia richiardii*	2	*Coquillettidia richiardii* (179); *Aedes annulipes* (1)	0.57	2.17	6.04	BOLD:AAI1618 (*Coquillettidia perturbans*)
BOLD: AAW9535	3	*Orthopodomyia pulcripalpis*	1	*Orthopodomyia pulcripalpis* (1)	0.00	0.00	4.97	BOLD:AAW9539 (*Orthopodomyia alba*)

Table 1 (continued)

BIN	Total BIN members	Taxa (this project)	Specimen count (this study)	Taxa included in the BIN (specimen count)	BIN mean intra-SP (%)	BIN max. intra-SP (%)	Distance to NN BIN (%)	NN BIN (taxa)
BOLD: ABY8239	698	*Anopheles messeae*	1	*Anopheles messeae* (228); *An. daciae* (90); *An. daciae* sp. inq. (376); *Anopheles* sp. (2); *Anopheles maculipennis* complex GB2013 (1); *An. maculipennis* s.l. BTLHVDV2014 (1)	1.57	4.19	1.33	BOLD:AAA9632 (*Anopheles maculipennis*)
		Anopheles daciae	4					
BOLD: ABZ7976	42	*Culex laticinctus*	2	*Culex laticinctus* (27)	0.50	1.36	1.76	BOLD:AAA4752 (*Culex theileri*)
BOLD: ACB9122	80	*Aedes sticticus*	21	*Aedes sticticus* (57); *Ae. rusticus* (1); *Ae. rossicus* (1)	0.31	1.28	1.70	BOLD:ACO9361 (*Aedes nigrinus*)
BOLD: AEG2154	21	*Aedes geniculatus*	7	*Aedes geniculatus* (20); *Aedes* sp. GW76 (1)	0.32	1.32	1.10	BOLD:AAM5898 (*Aedes geniculatus*)
BOLD: AEV9902	446	*Culex torrentium*	17	*Culex torrentium* (441); *Cx. pipiens* (2); *Cx. modestus* (1); *Aedes japonicus* (1)	0.37	2.74	1.04	BOLD:AAA4751 (*Culex pipiens*)

Intrudens Group (CROCU102-21) was recorded in the Pannonian-Peripannonian region (Klokočevci – Fig. 1) and represents new data not only for this region, but also for the Croatian fauna in general.

The success rate of COI amplification and sequencing from both directions in relation to the number of samples processed was 72.7% (197 / 271 specimens), while the percentage of successful amplification reactions of ITS2 was 98.5% (132 / 134 specimens of the *Anopheles maculipennis* complex). The remaining specimens were excluded from further analyses due to failed amplification or low sequence quality. A minimum of one and a maximum of 21 COI sequences were obtained per species, with an average of six.

The BIN-RESL algorithm in BOLD assigned the specimens to 31 BINs (Table 1), with most delineated MOTUs matching the morphological identification. Several BINs were discordant, with two or more species placed together. BIN discordance was present in the genus *Aedes* and the genus *Anopheles*, mainly in the species that were difficult to distinguish morphologically. These are the BINs with samples identified as *Ae. annulipes*, *Ae. cantans, Ae. riparius* and *Ae. excrucians* (BOLD:AAB1098); *Ae. rossicus* and *Ae. cinereus* (BOLD:AAP8897); *Ae. caspius* and *Ae. zammitii* (BOLD:AAB7911); *An. messeae* and *An. daciae* (BOLD:ABY8239). Barcode gap analysis in BOLD confirmed the presence of a clear barcoding gap within the public BOLD dataset DS-CROCU2 (Fig. 2). The concordance with the morphological identification was 98.48%.

In 13 species, the genetic distances of the species BIN to the nearest neighbour (NN) (i.e. non-specific specimen with the lowest interspecific distance in BOLD) were higher than the maximum intraspecific genetic distances, while other species had a lower value (Table 1). The mean intraspecific p-distance ranged from 0% to 1.68%, calculated for MOTUs delimited with BIN-RESL. The largest intraspecific divergence was observed in BIN BOLD:AAA7067 for the species *Ae. vexans* (7.22%), followed by BIN BOLD:AAB2483 for *An. hyrcanus* (5.32%) and BIN BOLD:AAA4751 for *Cx. pipiens* s.l. (5.02%). Only one species, *Or. pulcripalpis* (BOLD:AAW9535), showed no intraspecific variability in BOLD (Table 1).

The ASAP method delimited 28 MOTUs (Fig. 3). Some species of the genera *Culex, Aedes* and *Anopheles* were assigned to the same MOTUs, mostly again the species that are difficult to distinguish morphologically (Fig. 3). A very similar delimitation was achieved using the bPTP method, resulting in 30 MOTUs being assigned to our dataset. Using bPTP and BIN-RESL analysis, *An. claviger* s.s. was subdivided into two MOTUs. Also, *Cx. modestus* was subdivided into two MOTUs, but only using the bPTP delimitation method. The BIN-RESL method divided *Cx. torrentium* and *Cx. pipiens* s.l. into two different BINs, while ASAP and bPTP analyses classified them to the same MOTU.

Most of the morphologically identified species formed well-supported monophyletic groups in the ML tree, which mostly corresponded to the assigned BINs, with support values between 81 and 100% bootstrap / > 0.9 aLRT (Fig. 3). Several species appeared to be paraphyletic, including *Cx. pipiens* s.l. in relation to *Cx. torrentium* and *An. daciae* and *An. messeae* in relation to *An. maculipennis* s.s.

Of the 132 specimens morphologically identified as *Anopheles maculipennis* complex, three species were confirmed using the ITS2 sequence: *An. maculipennis* s.s., *An. messeae* and *An. daciae*. *An. maculipennis* s.s. was the predominant species with 56.06% of the samples, followed by *An. messeae* with 36.36% and *An. daciae* with 7.57% of the samples.

Based on both morphological and molecular identification, a total of 34 species were identified in this study (Table 1).

Discussion

This work represents the first comprehensive study aimed at generating DNA barcodes for the establishment of a DNA barcode library for the Croatian mosquito fauna. Based on morphological characteristics, 30 mosquito species were identified, divided into six genera, and four more species were confirmed based on molecular data, which in total represents about 63.5% of the total number of mosquito species recorded in Croatia [11]. The validity and accuracy of the data obtained was examined using species delimitation algorithms, with various clustering methods additionally supporting the MOTUs obtained. This study shows that, in addition to morphological classification, molecular taxonomy can also be a suitable tool for the identification and delimitation of mosquito species in Croatia, as has recently been shown for many other countries [36–38, 57, 62, 78, 79]. The concordance of morphological and molecular identification in this study is high, which is also consistent with recent similar studies [38, 62, 80]. Morphological and molecular data discrepancies might be due to misidentifications, resulting in inaccurate species names for sequences in public databases [81]. This study found minimal differences in species delimitation methods, mostly for morphologically indistinguishable species. The effectiveness of barcoding was confirmed by the presence of a clear barcoding gap, which is necessary for the effective application of DNA barcodes to identify specimens and delimit species [62, 82]. According to Meyer & Paulay [83], a barcoding

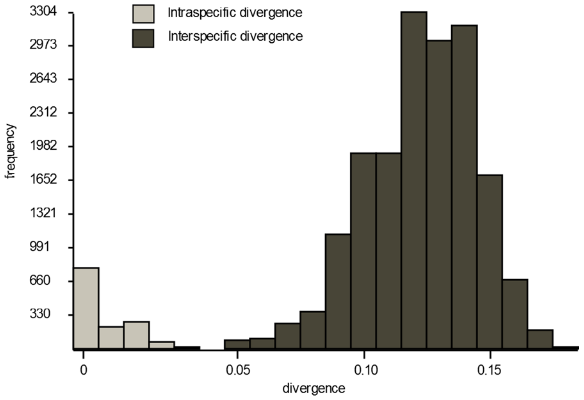

Fig. 2 Frequency histogram of p-distances within and between investigated mosquito species (MOTUs) for the public BOLD dataset DS-CROCU2

gap can be defined as the difference between the mean intraspecific sequence variability and the interspecific variability for congeneric COI sequences.

Based on DNA barcode analysis, most of the species morphologically identified in this study were categorised into established groups in ML tree analysis. A few individuals in certain taxa of the genera *Aedes* and *Anopheles*, such as *Ae. annulipes/cantans/excrucians*, *Ae. rossicus/cinereus*, *Ae. caspius/zammitii*, and *An. daciae/messeae*, were grouped within the same BINs, suggesting that the COI gene may not be informative enough for distinguishing among these species. This is also confirmed by the fact that these taxa occur in similar combinations in BOLD BINs. According to recent studies, these taxa are phylogenetically very closely related, although they have different morphological characteristics [38, 57, 62].

The *Anopheles maculipennis* complex consists of several species, seven of which have been recorded to belong to the Croatian fauna [11]. In this study three of them were identified, namely *An. maculipennis* s.s., *An. messeae* and *An. daciae*. In the COI sequence-based species delimitation, the *Anopheles maculipennis* complex was divided into two different MOTUs, *An. maculipennis* s. s. and *An. messeae/daciae*, by the results of the BIN-RESL algorithm. These two BINs are mutually nearest neighbours in BOLD, with only 1.33% distance between them, while in both BINs the highest intraspecific distance is about 4% (Table 1). The other two delimitation methods do not support this partitioning, as they group all *An. maculipennis/messeae/daciae* specimens into a single MOTU, with *An. messeae/daciae* specimens appearing as a paraphyletic group in the ML tree with respect to the *An. maculipennis* s.s. samples. This confirms the highly entangled situation in this species group which requires further investigation involving other molecular markers, similar as it was observed in Sedaghat et al. [84].

A rare species within the *Anopheles maculipennis* complex, *Anopheles melanoon*, previously recorded in the Croatian areas of southern Dalmatia and Istria [85], could not be confirmed in this study, possibly due to environmental modification of its distribution area. The occurrence of this species is closely linked to areas with horse and cow stables [86, 87], and such habitats have become quite rare. Other species within this complex previously detected in Croatia (*An. atroparvus*, *An. sacharovi* and *An. labranchiae*) [88, 89] were also not found in other recent surveys [50, 85]. Future studies should definitely focus on the detection of these species to confirm or exclude their current distribution in Croatia, as they are the most important malaria vectors in Europe [30].

The sibling species *Cx. torrentium* and *Cx. pipiens* s.l. can only be distinguished morphologically based on the characteristics of the male genitalia [67, 90]. The results of this study are consistent with the results of studies in Belgium [57] and show that these two species are separated as distinct MOTUs based on the BIN-RESL method, which was not the case in another recent study [62].

In contrast, the ASAP and bPTP methods did not show sufficient discrimination between species within genetically closely related species groups such as the *Culex pipiens* complex or the *Anopheles maculipennis* complex. For each of these two groups, the species are grouped into a single MOTU according to ASAP and bPTP.

The two sibling species *Anopheles claviger* s.s. (Meigen) and *Anopheles petragnani* Del Vecchio belong to the *Anopheles claviger* species complex [67], but *An. petragnani* has not yet been recorded in the Croatian fauna [11]. Within this species complex, COI has proven to be a sufficient tool to distinguish the two species [62]. Our two *An. claviger* s.s. specimens formed a strongly supported clade in the ML tree according to ASAP method, but were identified as separate MOTUs using bPTP and BIN-RESL methods. The specimen CROCU199-21 from a mountainous region was grouped in a BIN BOLD:AAM4220, where a wide European distribution with larger genetic distance (max. 3.56%) is observed. Another specimen, CROCU077-21 from the Mediterranean region, was grouped in BIN BOLD:AAE3979, with a narrower distribution (mainly Iran and Kosovo) and somewhat lower distances (max. 3.15%) (Table 1). A third BIN in BOLD with specimens from Tajikistan and China suggests that the species, *An. claviger* s.s., may be divided into several cryptic species. However, *An. petragnani* is placed in a separate BIN, including specimens from western Europe, some of them misidentified as *An. claviger*.

Fig. 3 ML tree based on COI DNA sequences of the specimens sequenced in this study. Numbers on branches denote bootstrap / aLRT support values (values lower than 70% are not shown). The results of species delimitation methods (bPTP, ASAP and BIN-RESL) are shown as vertical bars on the right

Fig. 3 continued

Culex modestus is considereda potential vector of WNV [91]. In this study, two individuals of *Cx. modestus* were detected in the Pannonian-Peripannonian region of Croatia near wetlands. This species is considered as the main vector of WNV in similar areas of southern France [92]. Although its dispersal ability is low, it could serve as an important enzootic and, given its ornithophilic and mammalophilic biting behaviour, bridging vector in natural/rural wetlands across Europe [67]. Therefore, in order to gain insights into the genetic structure of populations in this region, it should be studied in more detail in the future, using a larger number of individuals and localities.

Currently, there is no comprehensive database for all European mosquito species of the subgenus *Ochlerotatus*. According to Becker et al. [67], species groups associated with European species were classified according to region (Palaearctic, Fennoscandia, Germany and former USSR), which are also mentioned in our study (see below). The differences in the classification of species within each region will remain as long as there is no global analysis of the subgenus [67].

Within the Caspius Group there are six species, *Aedes berlandi*, *Ae. caspius*, *Aedes dorsalis*, *Aedes mariae*, *Ae. pulcritarsis* and *Ae. zammitii* [67], of which only *Ae. berlandi* has never been recorded in Croatia. According to all species delimitation algorithms used in this study, *Ae. caspius* and *Ae. zammitii* are grouped in the same MOTU, although they can be clearly distinguished morphologically. A similar case was observed in a recent study on the territory of Spain [80] for *Ae. mariae* and *Ae. caspius* which were grouped in the same MOTU. In our study the minimum interspecific distance between *Ae. zammitii* and *Ae. caspius* was 1.15%, similar to that in the aforementioned study [80], which is value lower than values typical between species [33]. Clearly, additional analyses of other loci that may better support species delimitation are required for a more accurate separation of species within this group.

Of the Annulipes Group, *Ae. annulipes, Aedes behningi, Ae. cantans, Ae. excrucians, Aedes flavescens* and *Ae. riparius* were recorded in Croatia [11, 67]. In general, distinction between species *Ae. annulipes* and *Ae. cantans* cannot always be accomplished with certainty based on morphological characters. Some sequences of the specimens morphologically identified as *Ae. annulipes*, *Ae. cantans* or *Ae. excrucians* matched the sequences in BIN: BOLD:AAB1098, which includes samples identified as *Ae. annulipes/cantans* or *Ae. excrucians/cantans*. One specimen, which had been assigned to the species *Ae. behningi* according to morphological characteristics, turned out to be *Ae. cantans* based on molecular analysis. In our ML tree, all specimens morphologically assigned to *Ae. annulipes* and *Ae. cantans* clustered into a single, well-supported clade, and all three species delimitation algorithms grouped the samples into a single MOTU. Consequently, COI proved to be an insufficient marker for distinguishing species within this species, as previously mentioned in other studies [38, 57].

In this study, the species *Ae. cinereus* and *Ae. rossicus* are grouped in the same MOTU according to all methods of species delimitation. Some samples morphologically identified as *Ae. cinereus* or *Ae. rossicus* were also confirmed based on COI sequences, while some could no be differentiated and had to be labelled as *Ae. rossicus/cinereus* (Fig. 3, Table 1). All individuals were collected in the Pannonian-Peripannonian region of Croatia. The taxonomic status of the species of the subgenus *Aedes* occurring in the Palaearctic is still unclear. *Ae. cinereus, Ae. rossicus* and *Aedes esoensis* were considered subspecies of the nominate form *Ae. cinereus* by Gutsevich et al. [93]. This view is not generally accepted, as *Ae. cinereus* and *Ae. rossicus* overlap strongly in Europe, the larvae often occur at the same breeding sites (which was also the case for our samples CROCU107-21, 108–21 and 109–21 from the same location and habitat—Fig. 3, Table 1), and transitional forms are unknown [67]. This is also supported by the fact that these two species are often found together, with samples from this study being assigned to the same BIN (BOLD:AAP8897).

A possible new species for the Croatian fauna, detected in this work, belongs to the Intrudens Group, which includes the species *Ae. diantaeus*, *Ae. intrudens* and *Ae. pullatus* [67]. Morphologically, the respective sample, CROCU102-21, was incorrectly determined as *Aedes punctor*, but after BIN-RESL analysis it was placed in BIN BOLD:AAF2904, which includes samples mainly identified as *Ae. intrudens* and *Ae. diantaeus*. This sequence matches 99.06% with the sequence of a specimen from Russia (KC855601) and 98.96% with a specimen from Sweden (JX040505), both of which were identified as *Ae. intrudens*. According to our photographs (available in the BOLD dataset DS-CROCU2) and the identification key according to Becker et al. [67], our specimen also corresponded to the description of the species *Ae. intrudens.* The eggs of this species overwinter, and the larvae are found from early spring onwards until the beginning of summer [67], which is consistent with our finding in April. In addition, the larvae of this species develop in temporary forest pools with dead leaves on the bottom [67], supporting our observations of its habitat. Considering that one single specimen was found, future studies should focus on the rediscovery and morphological and molecular confirmation of this species so that it can be reliably included in the list of Croatian mosquito species.

This six-year study covered 63.46% of the total mosquito fauna in Croatia. The distribution of species in

Croatia by region determined in this study essentially corresponds to the results of previous studies [11, 50, 51]. It should be emphasized that some species included in the currently valid Croatian mosquito species list [11] were last recorded a long time ago, such as species of the *Anopheles maculipennis* complex: *An. atroparvus*, *An. sacharovi* and *An. labranchiae* [88, 89, 94]. Efforts have been made to eliminate malaria mosquitoes over the past centuries, and habitat changes and pollution may have contributed to reducing their population or causing extinction in this area [95–97]. However, as these species are the main vectors of malaria in Europe [30], future research should focus on confirming their presence (or absence) in the area. Species that were not recorded in this study but had been recorded in some other recent studies [98–100] are monocyclic, rare or uncommon [67], such as *Aedes cataphylla*, *Aedes leucomelas*, *Ae. behningi*, *Ae. riparius*, *Culex martinii*, *Culiseta morsitans* and *Culiseta subochrea*. In addition, some species are linked exclusively to certain habitats and periods of occurrence, and there was a high probability that they would not be recorded in this type of research. It is necessary to focus on these rare and unrecorded species in the future so that they can be sampled specifically and thus expand the database of Croatian mosquito barcodes in BOLD.

Conclusions

With barcoding sequences for 34 Culicidae species, the results of this study represent the basis for the establishment of a reference DNA barcode library for mosquitoes in Croatia. It has been proven that barcoding is an appropriate tool for the additional identification and delimitation of species of the Croatian mosquito fauna, even for closely related species such as *Cx. pipiens* s.l. and *Cx. torrentium*, while ITS2 proved a suitable marker to differentiate closely related species within the *Anopheles maculipennis* complex. The inability of COI to distinguish certain biotypes or other closely related species (such as *Cx. pipiens* biotype *molestus*; *Ae. annulipes/cantans; An. messeae/ daciae*) needs to be compensated for by additional molecular markers. In the future, studies should focus on confirming the species from the Croatian mosquito fauna list that were not included in this study. For the discovered potentially new species of the Intrudens Group, targeted investigations involving sampling of individuals in all life stages are needed to substantiate their inclusion in the list of Croatian mosquito fauna. Rapid and accurate identification is a crucial step in mosquito surveillance and control, so the data here are important not only for the assessment of biodiversity and of the geographical distribution of potential vector species in the studied area. The reference barcode sequences will contribute to future research on the mosquito fauna in Croatia, neighbouring countries and Europe in general, facilitate the identification or detection of potentially misidentified or cryptic species and provide a basis for invasive and vector species surveillance and monitoring projects.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-024-06291-9.

Supplementary Material 1: Table S1. All samples analysed in this study with their BOLD IDs, divided by regions of Croatia where they were found.

Supplementary Material 2. Dataset S1. Nucleotide alignments of COI and ITS2 datasets for all analysed specimens.

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Author contributions
Conception and design of the work: NB, BBM, EM, MK; samples acquisition: NB, AK, NL, TŽ, GV, NT, MSB; analyses: NB, BBM; interpretation of data: NB, BBM, EM; manuscript draft: NB, BBM; manuscript revision: AK, EM; funding acquisition: MK, EM, NB.

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Availability of data and materials
All relevant data are within the manuscript and its additional files. All information about barcoded specimens can be found in the public BOLD dataset DS-CROCU2. The vouchers of barcoded specimens are kept in Josip Juraj Strossmayer University of Osijek, Department of Biology, and in Andrija Štampar Teaching Institute of Public Health in Zagreb. DNAs of barcoded specimens are kept in Josip Juraj Strossmayer University of Osijek, Department of Biology.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Biology, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia. [2]Andrija Štampar Teaching Institute of Public Health, Zagreb, Croatia. [3]Teaching Institute for Public Health of the Istrian County, Pula, Croatia. [4]Teaching Institute for Public Health, Split-Dalmatia County, Split, Croatia. [5]Teaching Institute for Public Health of the Osijek-Baranja County, Osijek, Croatia. [6]Faculty of Science, Department of Biology, University of Zagreb, Zagreb, Croatia. [7]Department of Molecular Biology, Ruđer Bošković Institute, Zagreb, Croatia.

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Publisher's Note

CHAPTER 4

Frequency of *Culex pipiens* complex members in continental Croatia

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FREQUENCY OF *CULEX PIPIENS* COMPLEX MEMBERS IN CONTINENTAL CROATIA

Nataša Bušić, Diana Lukić, Gabrijel Mirković, Ivana Skuzin & Enrih Merdić*

Josip Juraj Strossmayer University of Osijek, Department of biology, Cara Hadrijana 8/A,
31000 Osijek, Croatia

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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*Corresponding author: enrih@biologija.unios.hr

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; Dobrotworsky, 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 (Farajollahi *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).

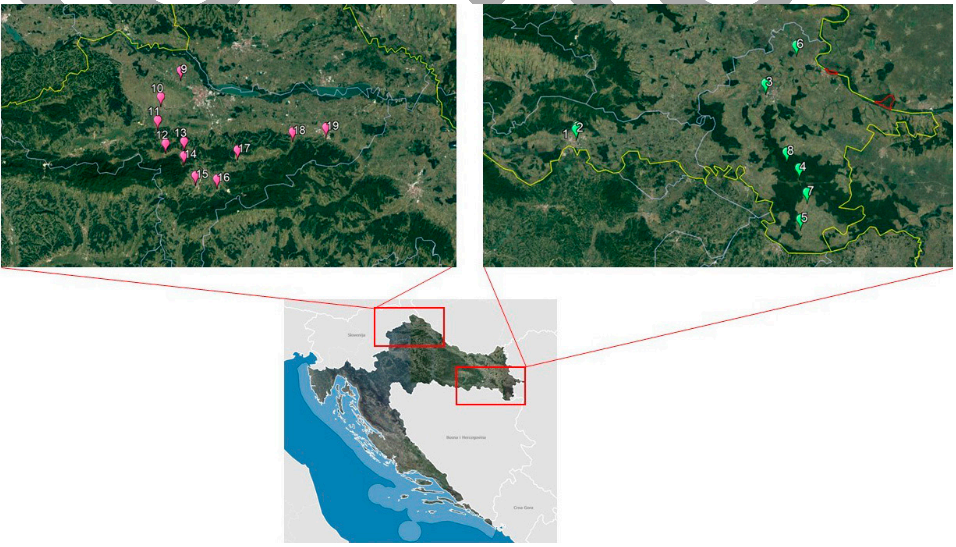

Fig. 1. Sampling sites of mosquitos in South East continental Croatia (green marks, 1-8) and in North West Croatia (pink marks, 9-19)

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 GenEluteTM 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 cordinates	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	*Cx. pipiens* s.l.	biotype *molestus* hybrid	barrel
2	Slavonski Brod	Brod-Posavina	03.06.2022.	45°09'58" N 18°01'57" E	96	*Cx. pipiens* s.l.	biotype *pipiens*	barrel
3	Vinkovci	Vukovar-Srijem	03.06.2022.	45°17'17" N 18°46'20" E	79	*Cx. pipiens* s.l.	biotype *pipiens*	channel
4	Spačva (Donje Novo Selo)	Vukovar-Srijem	03.06.2022.	45°03'15" N 18°54'00" E	85	*Cx. pipiens* s.l.	biotype *pipiens* hybrid	pound
5	Drenovci	Vukovar-Srijem	03.06.2022.	44°54'50" N 18°54'11" E	82	*Cx. pipiens* s.l.	biotype *pipiens*	barrel
6	Otok	Vukovar-Srijem	03.06.2022.	45°09'38" N 18°53'40" E	83	*Cx. pipiens* s.l.	biotype *molestus*	barrel
7	Vrbanja	Vukovar-Srijem	03.06.2022.	44°59'13" N 18°55'44" E	80	*Cx. pipiens* s.l.	biotype *pipiens*	barrel
8	Otočki Virovi	Vukovar-Srijem	03.06.2022.	45°05'55" N 18°51'08" E	78	*Cx. pipiens* s.l.	biotype *pipiens*	pound
9	Hrašćica	Varaždin	23.06.2022.	46°19'42" N 16°17'21" E	175	*Cx. pipiens* s.l.	biotype *pipiens*	barrel
10	Vidovec	Varaždin	23.06.2022.	46°17'11" N 16°14'41" E	179	*Cx. torrentium*	-	barrel
11	Tužno	Varaždin	23.06.2022.	46°14'59" N 16°14'20" E	185	*Cx. pipiens* s.l.	biotype *pipiens*	barrel
12	Završje Podbelsko	Varaždin	23.06.2022.	46°12'47" N 16°15'30" E	194	*Cx. pipiens* s.l.	-	barrel
13	Sveti Ilija	Varaždin	23.06.2022.	46°13'01" N 16°18'02" E	199	*Cx. pipiens* s.l.	biotype *pipiens*	concrete trough
14	Podevčevo	Varaždin	23.06.2022.	46°11'41" N 16°18'05" E	258	*Cx. pipiens* s.l.	biotype *pipiens*	barrel
15	Novi Marof	Varaždin	23.06.2022.	46°09'49" N 16°19'40" E	188	*Cx. pipiens* s.l.	biotype *pipiens*	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	*Cx. pipiens* s.l.	biotype *pipiens*	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.

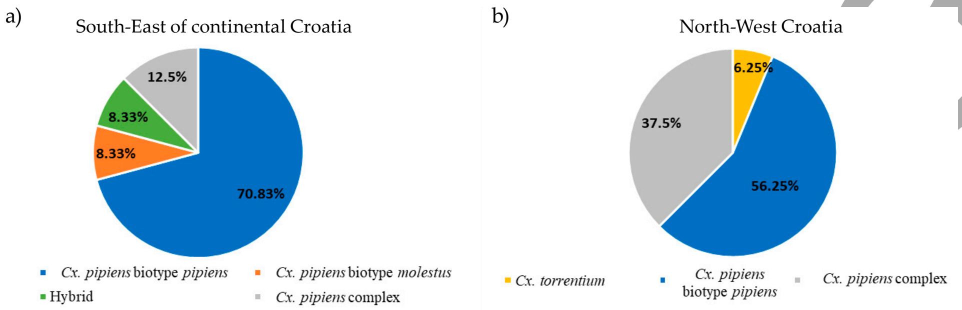

Fig. 2. Abundance of species in South East continental Croatia (a) and North West (b) Croatia in percentages.

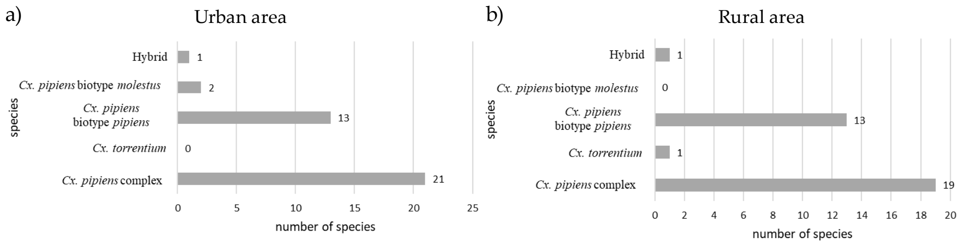

Fig. 3. Number of specimens in the urban (a) and rural (b) part of the research area.

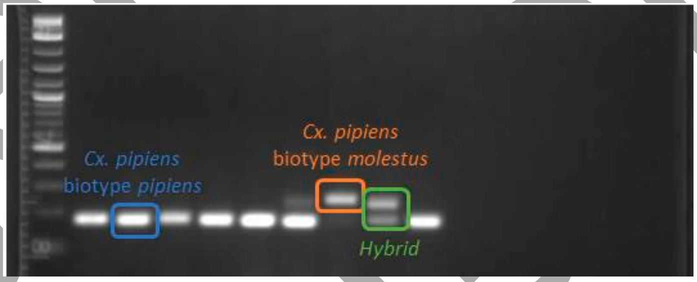

Fig. 4. Example of PCR amplification of the flanking region of the CQ11 microsatellite of *Culex pipiens* complex collected in South East of continental Croatia.

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-made 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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GENERAL DISCUSSION

Modern taxonomic and faunistic work requires an integrative, multidisciplinary approach that includes morphological, molecular and distributional/ecological data (Cywinska et al., 2006; Chan et al., 2014). In this dissertation, the results of a six-year integrative taxonomic study of the Croatian mosquito fauna are presented and discussed in terms of the importance of data on their distribution and vector competence.

Over the past two decades, 52 species have been recorded for the Croatian mosquito fauna, divided in eight genera (*Anopheles* - 12 species, *Aedes* - 24 species, *Coquillettidia* - one species, *Culex* - seven species, *Culiseta* - six species, *Orthopodomyia* - one species, and *Uranotaenia* - one species). Identification was mainly carried out by morphological methods (Merdić et al., 2020), while molecular analyses have been used only very sporadically. Only a few species have so far been confirmed using molecular markers, including *Culex torrentium* (Merdić et al., 2018) and several *Anopheles* species, *Anopheles messeae*, *Anopheles maculipennis* s. s., *Anopheles melanoon* and *Anopheles daciae* (Vignjević, 2014), emphasizing the need for further genetic and ecological studies. While the mosquito fauna of Pannonian-Peripannonian (Sudarić Bogojević et al., 2008; Merdić et al., 2010) and Mediterranean Croatia (Merdić et al., 2008; Romanović and Merdić, 2011) has been well studied, detailed research on the central mountainous region - where data was scarce and outdated (Merdić, 1995b; Janssen et al., 2020) - was conducted as part of this dissertation (Chapter 1). Although some species previously recorded in this region were not registered in this study (*Ae. cinereus*, *Ae. pulchritarsis* (Merdić, 1995b), *Cs. glaphyroptera* (Merdić et al., 2004) and *An. messeae* (Vignjević, 2014.)), several new species were collected. For the area of Gorski Kotar and Velebit these were the species *Cx. hortensis*, *Ae. punctor* and *Cs. longiareolata* (Chapter 1), and for the karst area of Lika *An. plumbeus*, *Cx. territans*, *Ae. sticticus*, *Ae. vexans* and the invasive species *Ae. albopictus* (Chapter 2). *Aedes albopictus* has a very well-established population in the Pannonian-Peripannonian and Mediterranean region in Croatia (Klobučar et al., 2024). Considering the vector competence of this species for 26 arboviruses (Paupy et al., 2009), it poses some risk to public health and the first detection of this species in this region suggests that its further spread is possible in the near future. In addition, some studies show that ecological niche and climate suitability models predict that many mountainous regions in southern and central Europe (e.g. parts of the Alps, the Dinaric Alps and the Balkans) may become increasingly favorable for the establishment of *Ae. albopictus* by 2050 and beyond (Roiz et al., 2011).

During the research in the Lika area, a large number of specimens of the invasive species *Aedes japonicus* were also found, especially on the border with Gorski Kotar. In previous studies in this area (Janssen et al., 2020), this species was only detected in two locations. These data indicate that the species is gradually spreading across Gorski Kotar to Lika and has adapted well to colder conditions and higher altitudes, as also found in similar studies, as well as to the possibility of coexistence with other species, especially those of the genus *Culex* (Chapter 2) (Cunze et al., 2016; Zielke et al., 2016; Montarsi et al., 2019; Janssen et al., 2020).

These data contributed to the knowledge of the fauna of the central mountainous region, which currently comprises 23 species, representing 44 % of all species recorded in Croatia to date. Numerous biotic and abiotic parameters such as temperature, humidity, geographical boundaries and the availability of vertebrate hosts influence the distribution range of a species. Although higher altitudes represent a natural barrier, these studies have shown that mosquitoes can also occur there in large numbers.

The eudominant taxa in this region was *Cx. pipiens* s. l., which was found at all altitudes, similar to the results of the recent study in Kosovo (Muja-Bajraktari et al., 2019), confirming its adaptability for development under different ecological conditions and at almost all types of water bodies (Amara Korba et al., 2016) (Chapter1). Their abundance near the inhabited houses, where we sampled many specimens of these taxa also explains and supports their popular name, the common house mosquito. The most common species in terms of abundance were *Cx. torrentium* in Gorski Kotar and Velebit areas (Chapter 1), and the invasive species *Ae. japonicus* in Lika (Chapter 2). As in previous studies in Europe (Aranda et al., 2000; Hesson et al., 2014; Merdić et al., 2018), this study also showed that the species *Cx. torrentium* prefers higher altitudes (in this case, the largest number of samples was recorded in the altitude range of 700 - 801 m a. s. l.), which is also confirmed by Poisson regression (Chapter 1). Unpublished research results in Croatia have shown that this species is widespread in all three regions, but is most abundant in the central mountainous region of Croatia (Bušić et al., under revision). In addition, previous publications have found that this species occurs in northern Europe up to a latitude of 46°N, but in southern Europe it only occurs at high altitudes (Hesson et al., 2014; Robert et al., 2019), a pattern that is consistent with its occurrence in Croatian mountainous areas such as Gorski Kotar and Lika. The interesting result shown in this study that this species can also be found at lower altitudes in Istria County (at 163 and 371 m a. s. l.) and in the Panonian-Peripannonian region (at 145 m a. s. l.), indicating that, contrary to previous studies, it does not only occur at higher altitudes in southern Europe (Hesson et al., 2014; Robert et al., 2019). The habitats in which the species was found were also quite interesting: a vase in a cemetery, a tree hollow, a canal and a barrel in a forest as well as a cellar. These findings suggest that *Cx. torrentium* may be adapting to warmer or Mediterranean climates, possibly with the help of favourable microhabitats such as shaded wetlands or cold- water springs.

In order to gain a better and more comprehensive insight into the mosquito fauna at higher altitudes, the mountainous part of the Pannonian-Peripannonian region of Croatia was also included (Chapter 1). The combined use of different diversity indices (Jaccard, Shannon-Wiener, Simpson, Evenness) enables a comprehensive understanding of mosquito distribution at different altitudes (Chapter 1, 2). The highest species richness (number of species) was found between 601 and 800 m a. s. l., and most specimens were collected between 501 and 800 m a. s. l., suggesting that this range provides optimal ecological conditions for several species. The most numerous were *Cx. pipiens* s. l., *Ae. japonicus*, *Cs. longiareolata and Cx. torrentium,* followed by *An. maculipennis* s. l., *Cx. hortensis*, *Ae. sticticus*, *Ae. geniculatus*, *An. plumbeus*, *Cx. territans* and, with a sample size of less than ten specimens, *Ae. vexans*, *Cs. annulata*, *Ae. cantans*, *Ae. communis*, *Cq. richiardii*, *An. claviger*, *An. plumbeus*. The Jaccard index confirms significant shifts in species composition with altitude, with greater similarity between sites within the same altitudinal range, as expected. The Shannon-Wiener index, which measures both species richness (number of species) and evenness (how evenly the individuals are distributed among the species), was highest at elevations 301 - 400 and 801 - 1200 m a. s. l., indicating a more even distribution of species and greater diversity. Although species richness was highest at altitudes of 601–800 m a. s. l., the Shannon-Wiener index was highest just below and above this range. This means that although species richness was lower at 301–400 and 801–1200 m a. s. l., species were more evenly distributed, i.e. no single species dominated these zones. On the other hand, many species are present in the altitude range 601–800 m a. s. l., but possibly a few very numerous species dominate (high species richness but lower evenness, lower Shannon than expected). In addition, Evenness index values were close to one at these elevations (301–400 and 801–1200 m a. s. l.), indicating that no species was overly dominant. The Simpson index was lowest at the same altitude range, indicating high diversity and low dominance of a single species. In contrast, the same indices showed a lower diversity and dominance of a single species in the altitude range from 501 to 800 m a. s. l. The Evenness and Simpson index supports greater diversity shown by the Shannon-Wiener index. The studied area was classified as warm and extremely warm with normal precipitation conditions, when the sampling was carried out (CMHS, 2017; 2020). These results emphasise the importance of altitude for the structure of mosquito communities and suggest that environmental changes may alter these patterns in the future. The use of multiple indices provides a more balanced picture when abundance and evenness are important.

Due to globalization and ecological changes, vector species are expanding their range, affecting the migration of birds and increasing the spread of arthropod-borne pathogens (Kirkpatrick and Randolph, 2012). Among other arboviruses, WNV has caused significant outbreaks in North America and parts of Central Europe (ECDC 2022; 2025; Ronca et al., 2021). In Croatia, human infections have been reported annually since 2012, with the major outbreak in 2018 (Vilibic-Cavlek et al., 2023). WNV transmission and epidemiology is influenced by various *Culex* taxa, with *Cx. pipiens* being the primary vector (Harbach, 2012; Vogels et al., 2017). Recent research suggests that a lesser known member of the genus *Culex*, *Culex* (Barraudius) *modestus*, may also play an important role in the transmission of pathogens, as it is thought to be more anthropophilic (bites humans) and may be potentially more competent than *Cx. pipiens* s. l. as a vector of West Nile virus (Soto et al., 2023). It would also be interesting to include the biology and behavior of this species, present in Croatia, in future research, as it can act as a vector and furthermore it showed high resistance to insecticides in a recent study (Soto et al., 2023).

Cx. torrentium and *Culex pipiens* biotype *pipiens*, which feed mainly on birds, maintain the enzootic cycle (Jansen et al., 2019), while *Cx. pipiens* biotype *molestus* feeds on mammals and rarely transmits WNV to humans. However, hybrids of *Cx. pipiens* biotype *pipiens*/*molestus* serve as bridge vectors and increase the risk of transmission (Hamer et al., 2008). Host preference is relative, with availability influencing the choice of host (Heym et al., 2019; Köchling et al., 2023). WNV also can overwinter if the infected females take a winter diapause (Dohm and Turell, 2001). In addition, in a recent study Usutu virus was detected in *Culex torrentium* individuals hibernating in the burrows of exposed animals, suggesting that this species may contribute to the persistence of the virus in Europe (Felix al., 2023).

Given the important role of these mosquitoes as vectors of diseases such as West Nile virus (WNV) and Usutu virus (USUV), these taxa were an important research target for this dissertation. A small part of the distribution and ecological characteristics of the *Culex pipiens* s. l./*Cx. torrentium* in Croatia is presented in the study on southeastern and northwestern continental Croatia (Chapter 4). Another comprehensive study covering all three geographical regions of Croatia has not yet been published (Bušić et al., under revision), but will be included in this discussion to provide a complete insight into the current distribution of these taxa based on the data collected and analyzed so far.

Until the research conducted as part of this dissertation, the biotypes of *Cx. pipiens* s. l. in Croatia were only mentioned in one paper (Merdić and Vuičić-Karlo, 2005), in which the presence of the *molestus* biotype in the Pannonian-Peripannonian region was demonstrated on the basis of the overwintering type. However, the results presented here are the first to use molecular methods for biotype identification within the *Cx. pipiens* s. l. in Croatia confirming the presence of *Cx. pipiens* biotype *pipiens*, *Cx. pipiens* biotype *molestus* and *Cx. pipiens* biotypes *pipiens*/*molestus* hybrids (Chapter 4; Bušić et al., under revision). The microsatellite CQ11, which was used in this dissertation as a molecular "ecotyping" method (Bahnck and Fonseca, 2006; Rudolf et al., 2013) to characterize the biotype of *Cx. pipiens* s. l., has proven to be a valuable tool for characterizing this species in Croatia, similar to other studies (Gomes et al., 2009; Reusken et al., 2010; Amraoui et al., 2012; Osório et al., 2014; Krida et al., 2015; Pan et al., 2025.). However, it should be emphasized that recent research (Haba et al., 2025) suggests that the use of CQ11 alone may be misleading, as the *molestus* - associated allele has been found in some *pipiens* populations due to variation in history or limited hybridization. The authors therefore suggest that, although CQ11 proved to be a useful marker, it should be interpreted with caution and complemented by genome - wide data or other lines of evidence (e.g. Kent et al., 2007; Rudolf et al., 2013). Our study has also shown that assays based on conventional PCR analysis not only have a higher failure rate (albeit for other samples), but are also more time-consuming than RT-qPCR (Bušić et al., under revision). In line with current knowledge, our future research will include more comprehensive genomic analyses of these taxa to gain more detailed insights into the representation and distribution of their biotypes.

Culex pipiens biotype *pipiens* was the most widespread taxon, occurring in all three biogeographical regions. This biotype dominates in the Pannonian – Peripannonian and Mediterranean regions with a higher percentage of occurrences in rural areas than in urban dwellings, which is supported by statistical significance (chi^2 test of homogeneity, p<0.05) (Bušić et al., under revision). Considering that it is a polyzonal and ecologically adaptable species (Vinogradova, 2000), which also occurs in similar habitats across Europe (Zittra et al., 2016; Hesson et al., 2014), this result was expected and also confirms the hypothesis of a higher prevalence of the *pipiens* biotype in rural areas. Studies conducted in Sweden, the Netherlands and Italy have shown that a higher proportion of the *pipiens* biotype is generally found in peri-urban habitats than on farms and wetlands, compared to *molestus* biotype and hybrids, which was also the case in this study. However, this pattern was not consistent across countries, suggesting that local environmental factors also play a significant role (Vogels et al., 2016). The occurrence of *Cx. pipiens* biotype *molestus* mainly in the Pannonian-Peripannonian region and in Mediterranean region (exclusively in Istria County) with only a few individuals in the central mountainous region indicates that this biotype is more widespread in north-eastern Croatia than in the south of the country.

In the present study, *Culex pipiens* biotype *molestus* was found mainly in rural environments (which is also supported by the statistical significance with the chi^2 test of homogeneity, $p<0.05$), similar to a study in Italy (Di Luca et al., 2016), where this biotype was detected above ground in natural and rural areas (Bušić et al., under revision). In contrast, in the narrower research area on the continental Croatia, this biotype was more likely to be found in urban areas (Chapter 4). Traditionally, this biotype is mainly associated with urban, enclosed and artificial underground habitats (Becker et al., 2020), but it can also be found in open agricultural landscapes (Osório et al., 2014; Zittra et al., 2016), again suggesting that local environmental factors play an important role in the distribution of biotypes.

When several cellars were sampled in similar habitats during overwintering of the total number of samples (n=25), only one *molestus* biotype and one hybrid specimens were found, while the rest were biotype *pipiens* specimens (Bušić et al., under revision). Both biotypes and their hybrids have been found in aboveground habitats across Europe (Vogels et al. 2016), which is also the case in our study, indicating that the *molestus* biotype is not restricted to belowground habitats as previously thought. This is supported by recent research using genomic evidence, which shows that the *molestus* biotype actually emerged thousands of years ago in the Middle East, adapted to above-ground human habitats associated with early agricultural civilizations, and only later colonized underground urban habitats such as subways and sewers (Haba et al., 2025).

Culex pipiens biotypes *pipiens*/*molestus* hybrids were detected in all three Croatian regions, with the highest percentage in the Mediterranean region and especially again in rural habitats (confirmed by chi^2 test of homogeneity, $p<0.05$) (Bušić et al., under revision), which does not confirm our hypothesis about a greater distribution of *molestus* biotype and hybrids in urban areas. Hybrid zones are typically peri-urban or agricultural areas, suggesting a spatial overlap in habitat use, especially in warmer climates where biotypes coexist above ground (Gomes et al., 2009; Zittra et al., 2016), which is also consistent with the results of our research. Both *pipiens* and *molestus* biotypes, along with their hybrids, were widely found in sympatry across various environments in whole country, similar as in other studies (Chevillon et al., 1995; Fonseca et al., 2004; Gomes et al., 2009; Reusken et al., 2010; Amraoui et al., 2012; Vogels et al., 2016). Moreover, the fact that members of *Cx. pipiens* complex can interbreed despite significant differences in physiology, ecology and behavior (Osório et al., 2014) indicates that reproductive isolation among members of the complex is not complete and that they can produce viable offspring under natural conditions (Yurchenko et al., 2020).

In general, it should be noted that rural areas predominate over urban areas in Croatia, and thus more rural and natural areas were sampled in the preparation of this dissertation. As a result, slightly more attention should be paid to urban areas in future studies. However, given the spread of invasive mosquito species in Croatia over the last decade (Vilibic-Cavlek. et al., 2023; Klobučar et al., 2024), citizens are becoming increasingly aware of the need to eliminate mosquito breeding sites in urban areas (Dobrača et al., 2024; Bušić et al., 2023), which could also have an impact on the number of mosquitoes sampled there.

Further research into these taxa should also be extended to the neighboring countries (Serbia, Bosnia and Herzegovina, Montenegro), from which some samples have already been collected and processed during this study. In Serbia the biotype *pipiens* has been confirmed so far, in Bosnia and Herzegovina hybrid, and in Montenegro, given the larger number of habitats sampled, both biotypes *pipiens* and *molestus*, as well as their hybrids and species *Cx. torrentium* (unpublished results). Expanding research on the *Culex* taxa in Croatia and neighbouring countries would lead to a better understanding of regional distribution and population dynamics, identification of the hybrid zones and predicting of potential hotspots for vector-borne diseases. Ultimately, it would have an impact on the improvement of the early warning systems and could thus support the coordinated mosquito control management and reducing of the cross-border spread of diseases.

The implementation of functional strategies to prevent vectors requires accurate mosquito identification. Although conventional identification using dichotomous keys is necessary, it has several limitations and is usually not accurate enough to identify species. Based on morphological characteristics, 30 mosquito species belonging to six genera were identified in this study (Chapter 3). Four additional species were validated by molecular analysis. These results emphasize the need and importance of a multidisciplinary approach to species identification (Cywinska et al., 2006; Chan et al., 2014). As in other countries, molecular analyzes have proven to be a sufficient tool to distinguish species, especially those that are cryptic and closely related (e.g. Hernández-Triana et al., 2019; Madeira et al., 2021). The same applies to the mosquito fauna of Croatia, where 63.5% of the total known species have been successfully barcoded. Various species delimitation algorithms and clustering techniques were used to validate the accuracy of the data and confirm the presence of a clear barcoding gap.

The majority of molecular analyses (98.58%) agreed with the morphological identification as in other similar studies (Madeira et al., 2021; Delgado-Serra et al., 2021; Chaiphongpachara et al., 2022). The finding of one specimen of a potentially new species for the Croatian fauna, belonging to the Intrudens Group (*Aedes intrudens*), further confirms the utility of this identification method. Initially, this particular specimen was identified as *Ae. punctor* based on morphological identification, but after BIN-RESL analysis, the specimen was identified as *Ae. intrudens*, and showed 99.06% similarity with the specimen of *Ae. intrudens* from Russia and 98.96% from Sweden. The ecology of the species and the habitat in which it was found matched the characteristics of the species according to Becker et al. (2020), demonstrating the value of the multidisciplinary approach to species identification. Since only a single specimen was detected in the present study, future research should aim to localise the species and confirm its identity through both morphological and molecular analyses, in order to ensure its reliable inclusion in the list of mosquito species in Croatia.

Cases in which COI has proven to be an insufficiently distinguishable genetic marker concern certain species of the genera *Aedes*, *Anopheles* and *Culex*, which has also been shown in other studies (Shahhosseini et al., 2020, Lamichhane et al., 2024, Hernández-Triana et al., 2019, Madeira et al., 2021). Several closely related species within the genus *Aedes* (e.g. *Ae. annulipes/cantans/excrucians*, *Ae. rossicus/cinereus*, *Ae. caspius/zammitii*) have indistinguishable COI gene fragment variations, which makes identification by DNA barcoding difficult. Khrabrova et al. (2013), for example, suggest relying on the morphological identification of fourth-stage larvae to distinguish between *Aedes* species.

Within the genus *Anopheles* there is a complex of sister species *Anopheles maculipennis*, which currently contains ten species (Linton, 2004). Since the members of this complex are all phylogenetically very closely related, it is difficult to distinguish them entirely on the basis of morphological characteristics, and the genetic marker COI also proved to be insufficient (Kronefeld et al., 2014; Hernández-Triana et al., 2019). However, another genetic marker, internal transcribed spacer 2 (ITS2) region from nuclear ribosomal DNA, proved to be sufficient for their differentiation (Di Luca et al., 2004; Madeira et al., 2021). Four species of the complex (*An. daciae*, *An. maculipennis* s. s., *An. melanoon* and *An. messeae*) have so far been confirmed molecularly in Croatia (Vignjević, 2014). During this six-year research, only *An. melanoon*, which was previously recorded, was not sampled. This information indicates the possible destruction of the very specific habitats to which this species is tied (Nicolescu et al., 2004; Linton et al., 2007). Furthermore, the species *An. maculipennis* s. s. was found in all three regions of Croatia, *An. daciae* in the Pannonian-Peripannonian and central mountainous region, while *An. messeae* was found only in the Pannonian-Peripannonian region. These results are consistent with those of previous studies, in which *An. messeae* was not found beyond 200 m above sea level, in contrast to *An. daciae* and *An. maculipennis* s. s., which can occur at higher altitudes (Kronefeld et al., 2014; Vignjević, 2014, Muja-Bajraktari et al., 2019). In contrast to COI barcoding, where *An. messeae* and *An. daciae* were often grouped into a single MOTU, ITS2 enabled differentiation of these two species, provided better resolution within the complex and showed low intraspecific variability. The intraspecific distances within the identified species remained low and ITS2 showed a high amplification success (98.5%), indicating its reliability as a genetic marker, similar to previous studies in other European countries (e.g. Nicolescu et al., 2004; Djadid et al., 2007; Simsek et al., 2011; Weitzel et al., 2012; Kronefeld et al., 2014; Kavran et al., 2018; Czajka et al., 2020; Smitz et al., 2021). On the other hand, the low genetic distance between *An. messeae* and *An. daciae* suggests recent divergence or ongoing localized gene flow (Brusentsov et al., 2023). Based on chromosomal and molecular differences from recent research, authors support recognizing *An. messeae* and *An. daciae* as separate species rather than as intraspecific forms (Brusentsov et al., 2023) and the results presented here also support this concept. However, further molecular markers are advisable to fully clarify the complex relationships within this species-group.

CONCLUSIONS

In accordance with the aims and hypotheses of the dissertation, the following conclusions were drawn.

This dissertation represents the first comprehensive analysis conducted to create a DNA barcode library for the mosquito fauna of Croatia. By combining conventional morphological identification with molecular taxonomy, it demonstrates the effectiveness of integrative approaches in accurately classifying mosquito species, including those that are morphologically similar or previously unrecorded. This work contributes significantly to our understanding of Croatian mosquito diversity and provides the basis for improved vector surveillance and control.

DNA barcoding confirmed the presence of 34 mosquito species in Croatia, potentially including the previously unrecorded species *Aedes intrudens*. DNA barcoding proved particularly useful for distinguishing closely related species such as *Culex pipiens* s. l. and *Cx. torrentium*, although additional markers are required for resolving complex taxa or other closely related species such as *Ae. annulipes*/*cantans* and *An. messeae*/*daciae*. The ITS2 marker proved reliable in distinguishing members of the *An. maculipennis* complex and confirmed the low intraspecific variability of the ITS2 region previously observed.

Species richness peaked in the mountainous regions between 601 and 800 m altitude, with diversity indices differing considerably at different altitudes. The occurrence of *Aedes albopictus* and *Aedes japonicus* in Lika and Gorski Kotar, including the first record of *Ae. albopictus* in central mountainous region, demonstrates the adaptability of invasive species to colder climates. This is an important finding for predicting future transmission risks of arboviruses such as dengue, Zika and chikungunya viruses as well as West Nile and Usutu viruses, which are mainly transmitted by *Culex* mosquitoes.

This study also provides the first molecular characterization of the biotypes of *Cx. pipiens* (*pipiens* and *molestus*) and their hybrids in Croatia using the microsatellite marker CQ11. The *pipiens* biotype was widespread, especially in rural areas, while *molestus* was more localized. Interestingly, the hybrids were most common in peri-urban and rural areas, contradicting the traditional view that they are mainly found in urban areas. Although CQ11 was informative, future studies should use genome-wide data to better clarify the differentiation of biotypes.

Altitude also influenced species composition. *Cx. pipiens* s. l. was more abundant at lower altitudes, while *Cx. torrentium* was more abundant at higher altitudes, although it was also detected at lower altitudes, expanding our understanding of its ecological range.

The results highlight the influence of environmental changes on the distribution of the species and emphasize the need for continuous monitoring. Future studies should: expand sampling to include urban areas more comprehensively; include genomic tools to gain deeper insights into the relationships between species and biotypes; research neighboring countries (Serbia, Bosnia and Herzegovina, Montenegro) where similar mosquito species and biotypes have been identified to enable coordinated regional control strategies and limit the cross-border spread of disease; conduct targeted studies on potentially new species such as *Ae. intrudens* at all life stages to confirm their taxonomic status and ecological role.

This dissertation makes a valuable contribution to entomological and public health research in Croatia by establishing a basic DNA barcode reference library for mosquitoes. The barcoded species include vectors of high medical relevance, such as *Aedes albopictus*, *Culex pipiens* and several *Anopheles* species. The study shows that while molecular methods such as DNA barcoding are powerful, a multidisciplinary approach combining morphological, molecular and ecological data is essential for accurate species delimitation and vector surveillance. The results will support more accurate public health planning and provide an important resource for ongoing mosquito surveillance in Croatia and the wider region.

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Curriculum vitae

Nataša Bušić (nee Graovac) was born on 4 May 1988 in Ljubljana, Slovenia. After attending Vladimir Nazor Primary School in Čepin and General Grammar School in Osijek, she graduated at Josip Juraj Strossmayer University of Osijek, Department of Biology, in 2014, with a Master of Education in Biology and Chemistry. She was the winner of the Lions Club Osijek Award for the best student at the Department of Biology in 2013. From 2014 to 2015 she worked as a biology and chemistry teacher at Vladimir Nazor Primary School in Čepin and from 2015 to 2017 as a nature and chemistry teacher at Ivan Filipović Primary School in Osijek. In May 2016, she passed the professional licence exam for the profession of biology and chemistry teacher. In the 2015/16 academic year, she enrolled in the doctoral programme in Nature and Environmental Protection in Osijek, which is jointly run by the Josip Juraj Strossmayer University of Osijek and the Ruđer Bošković Institute, Zagreb. Since March 2017, she has been employed as an assistant in the Department of Biology at the Institute of Zoology, where she still works today. In April 2017, she completed the WHO training on invasive mosquitoes of Europe. In addition to carrying out internships and teaching, she is involved in scientific and research-related work in the field of natural sciences, biology, zoology and didactics of natural sciences. She is the author of several scientific and professional articles in international and national journals and co-author of a book (list of all publications at: https://www.croris.hr/osobe/profil/35178). She actively participates in scientific and professional conferences at home and abroad as well as in various professional training programmes supporting lifelong learning. She is married and a mother of two boys.

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Editorial book:

Komarci Hrvatske / Merdić, Enrih (ur.). Osijek: Odjel za biologiju Sveučilišta Josipa Jurja Strossmayera u Osijeku, 2020

Institutional projects:

Barcoding of the mosquito fauna of Croatia with special emphasis on the polytypic species *Culex pipiens* complex, (UNIOS-ZUP 2018-55), collaborator

Comparison of the abundance and vectorial role of indigenous and invasive mosquito species in eastern Croatia, (3015-26-23), collaborator

Keep a bucket of water at bay to keep the sting away!– citizen science in Osijek-Baranja County area, (3105-5-21), collaborator

Determining the presence of arboviruses in mosquitoes in the Osijek-Baranja County area, (3105-26-21), collaborator

Invasive mosquitoes in Lika, (3105-5), collaborator

Croatian Science Foundation projects:

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