ROMANIAN ACADEMY

INSTITUTE OF BIOLOGY BUCHAREST

SUMMARY OF THE PhD THESIS

USING INTEGRATIVE TAXONOMY TOOLS TO IDENTIFY MOSQUITOES (DIPTERA, CULICIDAE) AS VECTORS OF DIFFERENT PATHOGENS IN THE MOST REPRESENTATIVE ECOSYSTEMS OF THE DANUBE DELTA AND CLUJ-NAPOCA AREA (ROMÂNIA)

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1.   INTRODUCTION

The estimation of arthropod diversity around human settlements becomes one of the major issues of medical entomology. Numerous human and animal diseases caused by pathogen microorganisms are transmitted by a large number of insect species therefore early detection is of growing importance in many European countries, including Romania. Mosquitoes are hematophagous insects, They could potentially transmit a series of pathogen organisms such us viruses (West Nile virus (Hubálek 2008)), bacteria (*Rickettsia* sp. (Socolovschi et al. 2012, Dieme et al. 2015)), protozoans (*Plasmodium* sp. (Piperaki & Daikos 2016)) and nematodes (*Dirofilaria* sp. (Kronefeld et al. 2014, Ionică et al. 2016)).

Globalization and climate change creates favorable conditions for several exotic pathogens to enlarge their original ranges toward temperate regions (Kilpatrick 2011, Lee et al. 2013). A series of recent works forecast re-emerging of once eradicated diseases in Europe or appearance of some new ones, because of an important supply of pathogens and their vectors like malaria mosquitoes from the surrounding regions (Africa or Asia) (Githeko et al. 2000, Hunter 2003, Lourenço et al. 2011). Malaria was detected in 2013 in Eastern Europe (Azerbaijan, Tajikistan and Turkey) where 2.9 million people are under permanent risk of being infected by this severe pathogen for human health (World Malaria Report, 2013). In 2015 there were 3.2 billion of people around the world exposed to the risk of malaria, with an estimated 214 million new cases and 438 000 deaths (World Malaria Report, 2015). Malaria is transmitted to people by species of mosquitoes belonging to genus *Anopheles*.

However other species of mosquitoes belonging to the Culicidae are able to inoculate a series of other pathogens (the so called mosquito-borne viruses or moboviruses) during their blood feeding activities on an infected donor and to deliver to a recipient host, including humans (Hubálek 2008). A series of recent works identified the repetitive presence of some moboviruses responsible for severe diseases in animals and humans which were once characteristic of only tropical areas (West Nile, Usutu, Dengue, Batai) (Hubálek 2008, Halstead 2008, Jöst el al. 2011, Becker et al. 2012, Roiz et al. 2012). All these data led to the development of an early warning system in many European countries. This was an important objective the monitoring and prevention of large scale epidemics (Bakonyi et al. 2007, Jöst et al. 2010, Becker et al. 2012, Allering el al. 2012). The presence of some moboviruses were also detected in Romania, like West Nile (Purcărea-Ciulacu 2008, Purcărea-Ciulacu & Nicolescu 2012), Tahyna, and Sindbis (Prioteasa 2011). The presence of several other new yet undetected moboviruses are expected, like Usutu and Dengue virus or Bunyaviruses, therefore the corroboration of these local data to the European Early Warning Systems is crucial to prevent local epidemics as well.

Introduction and spreading of moboviruses in some new geographic regions largely depends upon the presence of favorable habitats where the host mosquito species belonging to the Culicidae could develop large populations (Kilpatrick & Randolph 2012).

In addition, it should be mentionedthat other pathogens, such as nematodes, bring the risk of filariasis to 856 million people in 52 countries worldwide by mosquito transition. In 2000 over 120 million people were infected in the tropics and sub-tropics of Asia, Africa, the Western Pacific, and parts of the Caribbean and South America (World Filariasis Report,

2016). In Europe another pathogen is present in the form of the nematode *Dirofilaria* sp. which is a parasite transmitted primarily between dogs (or other canids which act as reservoir hosts) and mosquitoes, but which can also affect humans (Pampiglione et al. 2009).

For this reason, knowledge on Culicidae species, their distribution, population dynamics, feeding preferences (host species) and vector competence are of crucial importance in many countries in Europe, including Romania.

2.   AIMS OF THESIS

My PhD research is an integrative study about mosquitoes in two different ecosystems in Romania, focusing mostly on: **taxonomy** (1), **ecology** (2) and **parasitology** (3) of the identified Culicidae communities.

For our research we selected two different ecosystems:

Danube Delta Biosphere Reserve area was selected as one of the target wetland ecosystem for our research. This large natural ecosystem is the most representative mosquito breeding site in Romania and a highly used migration place for the birds. Four collection sites were selected (lake, marsh, forest and urban habitat), each having a representative number of samples from the mentioned area. This study is a longitudinal survey (2014-2015, April-September) focusing upon mosquito identification, their ecology preferences and their borne parasites.

The main objectives were:

1. Identification of Culicidae species used by morphology and genetic analyses

1. Phylogenetic analysis of Culicidae species

2. Characterization of mosquito species (spatial and temporal analysis, flight period, habitat and climate preference, modeling species distributions in Danube-Delta)

2. Comprehensive study on the mosquitoes' community (Diptera, Culicidae) from different habitats in Danube Delta

2. Diversity analyses on the mosquitoes' community (Diptera, Culicidae) from different habitats in Danube Delta

3. Molecular detection of mosquito-borne viruses from different Culicidae vector species

3. Molecular detection of Nematode species from different Culicidae vector species

Using the data from our research we would like to develop an early warning system for humans regarding the presence of these moboviruses in these ecosystems.

Cluj-Napoca is the second largest city in Romania, with of a population of more than 324600 people (in 2011) (www.population.city/romania/cluj-napoca/), but also with abundant semi-natural and artificial aquatic and wet ecosystems. In this area we focused a longitudinal survey (2015, April-September) to identify mosquito species and their mosquito-borne viruses.

The main objectives were:

1. Identification of Culicidae species

2. Characterization of mosquito species

3. Molecular detection of mosquito-borne viruses from different Culicidae vector species

3. MATERIAL AND METHODS

3.1. Material collected and examined in Danube Delta Biosphere Reserve

Figure 1. Figure 1.Our longitudinal studies involved two consecutive years 2014-2015, between April and September. Four representative mosquito trapping sites were selected in the Danube Delta Biosphere Reserve (DDBR) within an area of about 160 km^2 and a minimal linear distance of ten kilometers between the sites. Research permits and approval (ARBDD 15/25.04.2014, ARBDD 16/24.04.2015) were issued by the Danube Delta Biosphere Reserve Authority. The season's collections being carried out from a total of four locations, four different ecosystems: forest-, marsh-, urban- and aquatic ecosystem (Figure 1.).
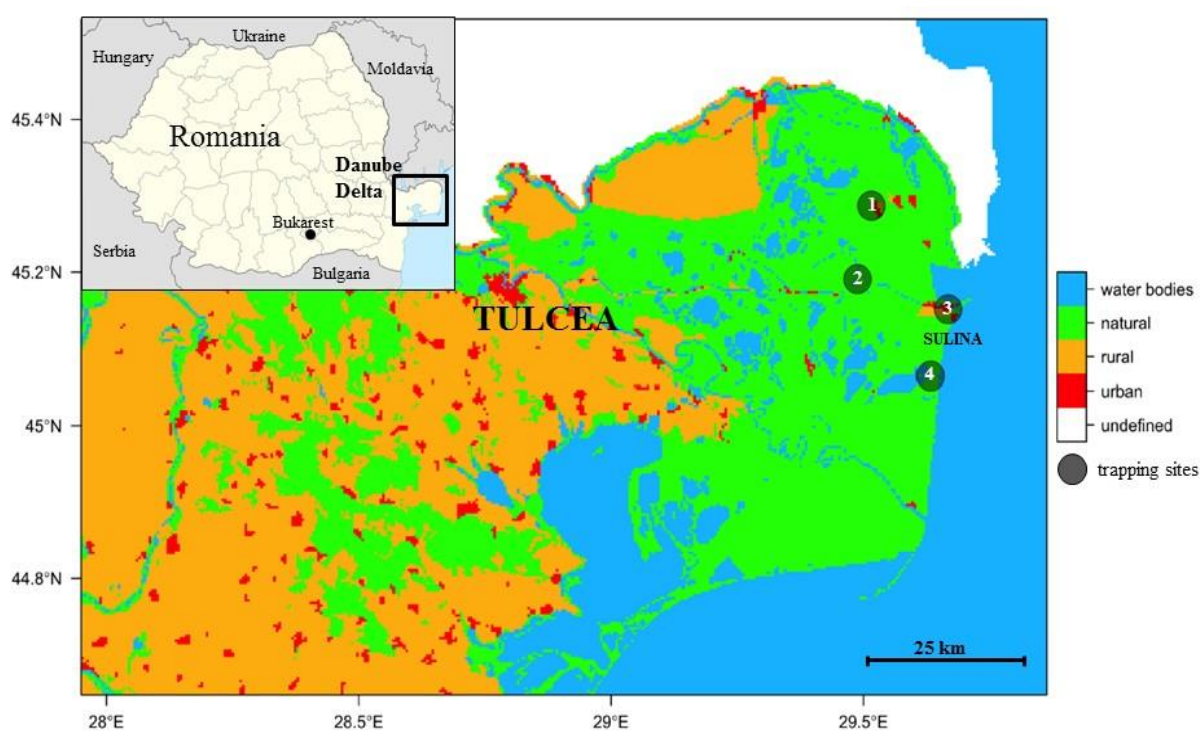

Figure 1. Collection sites in Danube Delta (1. forest ecosystems; 2. marsh ecosystem; 3. urban ecosystem; 4. aquatic ecosystem)

We used carbon dioxide-baited Heavy Duty Encephalitis Vector Survey traps (EVS, Bioquip Products Inc., California, USA), using standard protocols (Šebesta et al. 2010). The traps consist of a 3.5l isolated plastic dry ice container with holes at the lower part of the container to allow the CO2 to escape. In the middle part of the trap a tube with a fan is located which are powered by three 1.5 V batteries. The mosquitoes are sucked downwards by the airflow and are blown in a nylon netting catch bag (Jöst et al. 2010). EVS traps collect a large number of mosquitoes of different species, which could not be caught with other traps. At each site 5 EVS traps are placed at about 15 m apart from each other. In all these four ecosystems the EVS traps operated during the maximum activity period of mosquitoes from 16.00 pm to 10.00 am the following morning. Living mosquitoes were preserved on dry ice prior to identification.

3.2. Material collected and examined in Cluj-Napoca

We carried out a survey to detect Culicidae communities from different semi-natural and wet ecosystems from the city of Cluj-Napoca in 2015. Mosquitoes were sampled from May to September (17 weeks), from 7 study sites from different wet or humid ecosystems, as follows (Figure 2.):

1. The Someșul Mic river side near "Eastern Hills of Cluj" hayfields, GPS coordinates 46°47'22.74"N, 23°36'1.47"E

2. Riparian vegetation along a secondary channel of Someșul Mic river near "Eastern Hills of Cluj "hayfields, GPS coordinates 46°48'2.80"N, 23°36'24.38"E

3. "Lacul 3" urban lake, GPS coordinates 46°46'28.85"N, 23°37'48.58"E

4. Fishing lake, GPS coordinates 46°46'40.34"N, 23°38'44.34"E

5. Water Treatment Station in center of Cluj-Napoca, GPS coordinates 46°45'53.05"N, 23°33'4.56"E

6. Botanical Garden, GPS coordinates 46°45'41.49"N, 23°35'10.93"E

7. Forest habitat in "Făgetul Clujului – Valea Morii, GPS coordinates 46°45'25.44"N, 23°33'52.94"E

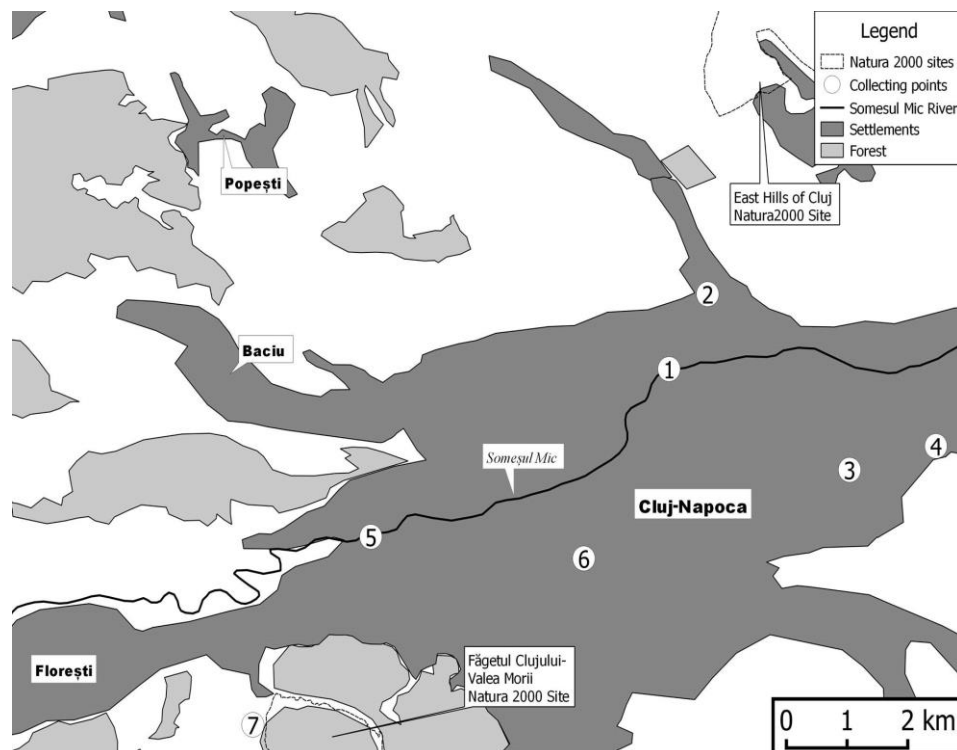Figure 2. Collection sites from Cluj-Napoca.

We used handmade Gravid Traps (GT), following the standard protocols (Scott et al. 2001, Williams & Gingrich 2007). The GT traps are designed to catch mostly gravid female mosquitoes, during their search for ovipositioning sites. The traps were equipped with a dark colored open-topped container filled with a water-based infusion. Above the water surface, a

fan was fixed in order to produce an upward air circulation. 4 D/Mono batteries with 1.5 V were used as power supply. Gravid females attracted by the decaying material placed in the infusion and open water surface were sucked in the net and collected regularly.

Taxonomical methods

Culicidae species were identified based upon morphological characteristics using identification keys by Becker et al. 2010 and Kenyeres & Thót, 2008 as well as on identification program software by Schaffner et al. 2001 'The mosquitoes of Europe: an identification and training program'. Specimens were examined with an Olympus SZ50 microscope, and the specimens were identified using ice as substrate. For virus analysis, we stored in the freezer in -80C°. All collection data are available on the TransDiptera Online Database (Kolcsár et al. 2018).

The morphological identification of these specimens was confirmed by the analysis of the mitochondrial cytochrome oxidase subunit I (COI) gene (Versteirt et al. 2015, Batovska et al. 2016). The resulting COI barcodes were used to corroborate the morphometric results with the molecular data (Török et al. 2016).

In order to investigate the phylogenetic relationship between different mosquito species collected during our study with those previously reported worldwide and available from GenBank, a maximum likelihood (ML) analysis was performed using PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/versions.php) with 1,000 pseudoreplicates. To assess the robustness of ML phylogenetic groupings, a bootstrap resampling analysis was conducted using 1000 replicate neighbour-joining (NJ) tree and Tamura-Nei distance model (TN93) in MEGA7 (Kumar et al. 2016). Sequences were deposited in GenBank with the accession numbers KU214640-KU214675 and KT876464-KT876495 (Török et al. 2016).

In additional we created a Romanian Culicidae Library, summarizing all Culicidae sequencing from Romania in Gen Banks and using DNA Sequence Polymorphism Analysis for detected haplotypes by DnaSP v5 software (Rozas et al. 2017).

Ecological methods

Statistical analyses were performed with RStudio version 1.1.442 (RStudio Team 2015) and PAST version 3.19 statistical environment (Hammer et al. 2001). We used the following analysis for compare collection sites: Principal Component Analysis (PCA), Discriminant Analysis (DA), Hierarchical cluster analysis, MANOVA analyses, Rarefaction analysis, Diversity analyses. We demonstrate the flight period based upon two collection years (Wang et al. 2015). For species distribution we used an Environmental niche model by Maxent (v 3.4.1.) For the species ecological preferring we used linear models Bayesian method within the MCMCglmm model was used in a general manner with Poisson distribution (Hadfield 2010) We used the following R packages: AICcmodavg, lme4, car, MCMCglmm (Hadfield 2010, Mazerolle 2017).

Parasitical methods

We udes the classical pan-PCR method was applied to detect members of families or genera of **moboviruses** (Flaviviridae, Orthobunyaviridae, Alphavirus, Rhabdoviridae and

Phebovirus). Another mobovirus identification tool was infection of mosquito Aedes albopictus clone cell cultures with viruses. The cell cultures used were *Ae. albopictus* cell-line C6/36 (Aag II, U 4.4 cell) (Igarashi 1978, Condreay & Brown 1986, Mizutani et al. 2003, Brackney et al. 2010) with an impaired RNAi pathway. After infection we incubate mosquito cell cultures, and after the multiplication of viral genome we amplified and sequenced using adequate protocols.

Nematodes identification were in all samples of mosquitoes collected in 2014 were pooled per sampling site, date and mosquito taxon, resulting in pools between 1 and 250 specimens (mean = 113.58). DNA of the mosquito pools was extracted and analyzed for the presence of *Dirofilaria repens* and *Dirofilaria immitis* DNA using a previously described duplex real-time PCR assay targeting nucleotide differences within the cytochrome c oxidase subunit 1 and 16S rRNA gene fragment (Șulesco et al. 2016).

4. RESULTS

4.1 Faunistic research of Culicidae in Romania

The first checklist of the Culicidae fauna of Romania was published in 1995 by Nicolescu. He presented 50 Culicidae species and additionally 6 records with uncertain data. We updated this checklist and increased the number of species to 60. Our results will be published paper Török et al. 2018. Including a synthesis of our faunistic results of the last few years focused mainly on some less studied areas in Romania such as Transylvania, from where new faunistic data on 20 different Culicidae species were recorded. Faunistic data based on morphological identification of the adult Culicidae were completed with 314 sequence data on 19 different mosquitoes species present in the countries fauna, downloaded from international genetic databases, but also based on sequences generated by the authors. This first DNA barcode library will be completed in the future; covering all the missing 41 species recorded from Romania and will help to a rapid identification of mosquitoes as well as adult-larvae associations from here.

We found a total number of 314 sequences belonging to 19 different Culicidae species (representing only 32% of the total Culicidae species from Romania) for four genes regions: 18S ribosomal RNA, NADH dehydrogenase 5 - MT-ND5 gene, mitochondrial cytochrome c oxidase I (mtCOI) and mitochondrial cytochrome c oxidase II (mtCOII). We found 75 haplotypes, with haplotype diversity 0.8764, selected region was 846 bp and 270 number of variable sites (Table 1.).

Table 1. Summarizing information of GenBank about Culicidae sequences from Romania species

Species	Gen Bank Cod	Gene	Authors	Haplotype
Aedes albopictus	HF536719.1	18S ribosomal RNA	Prioteasa,L., Dinu,S., Falcuta,E., Oprisan,G., Gatej,R., Badescu,D. and Ceianu,C.	1
Aedes albopictus	LN808745.1-LN808746.1, HF566374.1, HF912379.1, HF536717.1	Mitochondrial COI	Prioteasa,L., Dinu,S., Falcuta,E., Oprisan,G., Gatej,R., Badescu,D. and Ceianu,C./Falcuta,E., Dinu,S., Prioteasa,F.L., Gatej,R.I., Dikolli,E. and Ceianu,C.S.	1

Species	Accession numbers	Gene	Authors	Count
Aedes albopictus	LN808747.1-LN808748.1, HF566375.1, HF912380.1,HF536718.1	MT-ND5	Prioteasa,L., Dinu,S., Falcuta,E., Oprisan,G., Gatej,R., Badescu,D. and Ceianu,C./Falcuta,E., Dinu,S., Prioteasa,F.L., Gatej,R.I., Dikolli,E. and Ceianu,C.S.	1
Aedes cinereus	KT876487.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	1
Aedes vexans	KT876471.1, KT876477.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	2
Anopheles algeriensis	KU214665.1-KU214675.1, KT876491.1, KT876482.1, KT876467.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	13
Anopheles atroparvus	AY634505.1-AY634534.1	5.8S ribosomal RNA	Nicolescu,G., Linton,Y.-M., Vladimirescu,A., Howard,T.M. and Harbach,R.E.	1
Anopheles daciae	AY634406.1-AY634503.1	5.8S ribosomal RNA	Nicolescu,G., Linton,Y.-M., Vladimirescu,A., Howard,T.M. and Harbach,R.E.	1
Anopheles daciae	AY757934.1-AY757936.1, AY757954.1	Mitochondrial COI	Nicolescu,G., Linton,Y.-M., Vladimirescu,A., Howard,T.M. and Harbach,R.E.	4
Anopheles hyrcanus	HQ197433.1-HQ197436.1, KT876466.1	Mitochondrial COI	Toty,C. and Alten,B./ Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	5
Anopheles hyrcanus	HQ197460.1-HQ197463.1	Mitochondrial COII	Toty,C. and Alten,B.	3
Anopheles hyrcanus	HQ197510.1-HQ197516.1	5.8S ribosomal RNA	Toty,C. and Alten,B.	1
Anopheles maculipennis	AY634535.1-AY634566.1, AY579401.1	5.8S ribosomal RNA	Nicolescu,G., Linton,Y.-M., Vladimirescu,A., Howard,T.M. and Harbach,R.E./ Vladimirescu,A.F., Coipan,C.E., Nicolescu,G.M., Ilina,L., Bordea,S. and Purcarea Ciulacu,V.	1
Anopheles melanoon	AY634504.1	5.8S ribosomal RNA	Nicolescu,G., Linton,Y.-M., Vladimirescu,A., Howard,T.M. and Harbach,R.E.	1
Anopheles messeae	AY648982.1-AY648998.1, EF090197.1	5.8S ribosomal RNA	Nicolescu,G., Linton,Y.-M., Vladimirescu,A., Howard,T.M. and Harbach,R.E./ Danabalan,R., Ponsonby,D.J. and Linton,Y.-M.	3
Anopheles messeae	KU214662.1-KU214664.1, KT876469.1, KT876481.1, KT876489.1, AY757922.1-AY757933.1, AY757937.1, AY757938.1, AY757942.1, AY757950.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R./ Nicolescu,G., Linton,Y.-M., Vladimirescu,A., Howard,T.M. and Harbach,R.E.	6
Coquillettidia richiardii	KT876478.1, KT876472.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	2
Culex modestus	KU214650.1-KU214660.1, KT876488.1, KT876486.1, KT876479.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	6
Culex pipiens	KU214640.1-KU214649.1, KT876492.1, KT876490.1, KT876483.1, KT876480.1, KT876470.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	6
Culiseta annulata	KT876473.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S.,	1

			Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	
Ochlerotatus caspius	HM535249.1-HM535251.1	Mitochondrial COII	Porretta,D., Canestrelli,D., Urbanelli,S., Bellini,R., Schaffner,F., Petric,D. and Nascetti,G.	3
Ochlerotatus caspius	HM535293.1-HM535297.1, KT876468.1	Mitochondrial COI	Porretta,D., Canestrelli,D., Urbanelli,S., Bellini,R., Schaffner,F., Petric,D. and Nascetti,G./ Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	5
Ochlerotatus detritus	KU214661.1, KT876465.1, KT876476.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	1
Ochlerotatus flavescens	KT876484.1-KT876485.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	2
Ochlerotatus hungaricus	KT876474.1-KT876475.1, KT876493.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	3
Uranotaenia unguiculata	KT876494.1	Mitochondrial COI	Torok,E., Tomazatos,A., Cadar,D., Horvath,C., Keresztes,L., Jansen,S., Becker,N., Kaiser,A., Popescu,O., Schmidt-Chanasit,J., Jost,H. and Luhken,R.	1

From the 60 species of Culicidae species recorded from Romania, three species have no genetic references in any international Gene Banks. These species are: *Ochlerotatus duplex* (Martini, 1926), *Culex martinii* Medschid, 1930, *Coquillettidia buxtoni* (Edwards, 1923). Further investigations are recommended to complete this database with more complete data (Török et al. 2018). The DNA barcode Library initiated by the authors has a more practical importance, serving as a base to develop more effective mosquito control programs in the future.

The mosquitoes community from the Danube Delta Biosphere Reserve

Our results based on an extensive faunistic and taxonomy investigation resulted in more than half a million (538 617) specimens belonging to 17 species. In addition, we detected two new species to the Romania Culicidae fauna: *Anopheles algeriensis* Theobald, 1903 and *Ochlerotatus hungaricus* (Mihályi, 1955), which both have morphologically and also genetically been confirmed. Detection of these new species highlights the lack of knowledge about the composition and genetic diversity of the mosquito fauna in Romania and in the DDBR in particular. We sequenced 64 specimens, and compiled for the first time the Romanian Culcidae DNA Barcode Library. Our data from two consecutive vegetation periods (2014-2015) provide a first and detailed overview of the mosquito community in the DDBR. Monitoring of Culicidae through faunistic data provided to be important to identify potential vector species of disease and therefore acts as an early warning system for the region investigated by us.

Culicidae species identified in the present study:

Genus *Anopheles* Meigen, 1818
Subgenus *Anopheles* Meigen, 1818
1. *Anopheles algeriensis* **Theobald, 1903**

2. *Anopheles hyrcanus* (Pallas, 1771)
3. *Anopheles maculipennis messeae* (Falleroni, 1926)
Genus *Aedes* Meigen, 1818
Subgenus *Aedes* Meigen, 1818
4. *Aedes cinereus* Meigen, 1818
Subgenus *Aedimorphus* Theobald, 1903
5. *Aedes vexans* (Meigen, 1830)
Genus *Culex* Linnaeus, 1758
Subgenus *Culex* Linnaeus, 1758
6. *Culex modestus* Ficalbi, 1890
7. *Culex pipiens* Linnaeus 1758
8. *Culex torrentium* (Martini, 1925)
Subgenus *Neoculex* Linnaeus, 1758
9. *Culex martinii* Medschid, 1930
Genus *Culiseta* Felt, 1904
Subgenus *Culiseta* Felt, 1904
10. *Culiseta annulata* (Schrank, 1776)
Genus *Ochlerotatus* Lynch Arribalzaga, 1891
Subgenus *Ochlerotatus* Lynch Arribalzaga, 1891
11. *Ochlerotatus caspius* (Pallas, 1771)
12. *Ochlerotatus detritus* (Haliday, 1833)
13. *Ochlerotatus dorsalis* (Meigen, 1830)
14. *Ochlerotatus flavescens* (Müller, 1764)
15. *Ochlerotatus hungaricus* (Mihályi, 1955)
Genus *Coquillettidia* Dyar, 1905
Subgenus *Coquillettidia* Dyar, 1905
16. *Coquillettidia richiardii* (Ficalbi, 1889)
Genus *Uranotaenia* Lynch Arribalzaga, 1891
Subgenus *Pseudoficalbia* Theobald, 1912
17. *Uranotaenia unguiculata* Edwards, 1913

The mosquitoes community from the Cluj-Napoca

The total number of 728 Culicidae individuals collected by us are important in respect to the short period of our investigation, limited mostly to summer time (from June to September in 2015) and resulting in a number of 14 Culicidae species (Table 2.), which represents 46.66% of species identified from Transylvania. This is a relatively high number compared to recent report of Nicolescu et al. (2002, 2003a, 2003b, 2003c), who identified 30 Culicidae species in Transylvania. *Coquiuettidia richiardii* is reported from here for the first time from Transylvania. Another eight species are collected first time around Cluj-Napoca.

Table 2. Culicidae species identified in the present study, with numbers of individuals, sites and months.

Culicidae species	Number of specimens female/male	Collecting site	Collecting months 2015
Anopheles (Anopheles) claviger (Meigen, 1804)	16f	3	September
Anopheles (Anopheles) maculipennis complex Meigen, 1818	157f,2m	2, 7	July, August
Culex (Culex) pipiens Linnaeus, 1758	164f/154m	1, 2, 3, 4, 5, 6, 7	May, June, July, August, September
Culex (Culex) territans Walker, 1856	75f	1, 3, 4, 6	June, July
Culiseta (Culiseta) alaskaensis (Ludlow, 1906)	11f	4	September
Culiseta (Culiseta) annulata (Schrank, 1776)	11f,3m	4	July, August, September
Aedes (Aedes) cinereus Meigen 1818	10f	4	September
Aedes (Aedes) vexans (Meigen, 1830)	13f	4	June

Ochlerotatus (Ochlerotatus) annulipes (Meigen 1830)	3f,2m	3	August
Ochlerotatus (Ochlerotatus) cantans (Meigen, 1818)	3f, 3m	7, 4	June
Ochlerotatus (Ochlerotatus) caspius (Pallas, 1771)	9f	7	September
Ochlerotatus (Ochlerotatus) cataphylla (Dyar, 1916)	5f	3, 4	September
Ochlerotatus (Ochlerotatus) punctor (Kirby 1837)	32f, 5m	7	June
Coquiuettidia (Coquillettidia) richiardii (Ficalbi, 1889)	48f,2m	1, 3, 4	July, August, September

4.2 Taxonomy

Danube Delta Biosphere Reserve

In order to avoid wrong mosquito species identification and overlooked possible cryptic species, we combined the morphology-based identification with DNA-barcoding methods. The analysis of the intraspecific sequence variation (3.1 %) of the *An. algeriensis* COI gene revealed the existence of at least three new relatives. This result was supported by the phylogenetic analysis suggesting the occurrence of a heterogeneous *An. algeriensis* population within a relatively small region. Such differentiation might be especially important if different subpopulations may have different vector competence (Hardy et al. 1983). The congruence between morphology-based identification and DNA-barcode grouping based on phylogenetic clustering with high bootstrap support (≥ 95 %) was found for all morphologically identified taxa. The main difficulty in the phylogenetic tree reconstruction was the unbalanced amount of available nucleotide sequences from other countries. However, the mitochondrial gene (COI) based phylogeny clearly related the DDBR mosquito species to those collected in other European countries and gave evidence of population subdivision for the species *An. algeriensis* and *Oc. detritus*. Such differences suggest allopatric speciation evolvement or mixing of different mosquito populations, which developed in distinct geographic regions. Another interesting point worth mentioning here is the phylogenetic clustering of *Oc. hungaricus*. Although it seems to be a homogenous species, almost identical with the reference specimen from Hungary, further studies on genetic diversity of this rare species from other countries are necessary for a final assessment (Török et al. 2016).

Results of the mtCOI phylogenetic analysis were similar with a study of Harbach 2007. In our phylogenetic tree Culicidae form a monophyletic group. All major genera are separated from each other with highly supported ML bootstrap values at 100% replicates. Our molecular phylogenies are in concordance with the conventional recognition of two subfamilies of Culicidae Anophelinae, Culicinae and seven genera. We identified two groups; the first one is a cluster incorporating the closest related genera of *Coquillettidia* and *Anopheles*, and the second one with *Culiseta*, *Aedes*, *Ochlerotatus* and *Uranotaenia*, *Culex* species. *Culiseta*, *Aedes* and *Ochlerotatus* are sister groups in our phylogenetic tree. *Uranotaenia* is the closest neighbour of *Culex* genus. Culicidae relationshipswere generated with the maximum likelihood method of phylogenetic tree with estimation from mtDNA COI. High posterior probability (PP=1) has in *Culex* species *Cx. modestus* and *Cx. pipiens*. Nearest relative is *U. ungurculeata* (PP=1). *Cq. richiardii* is well separated from *Anopheles* species (PP=1). *An. algeriensis* are clustered together with *An. darlingi*, and are well separated from all other *Anopheles* species included in this study. The *Aedes* species are clustered together beingwell separated from a clade consisting of two subclades, *Ochlerotatus* and *Culiseta*. The subclade formed by
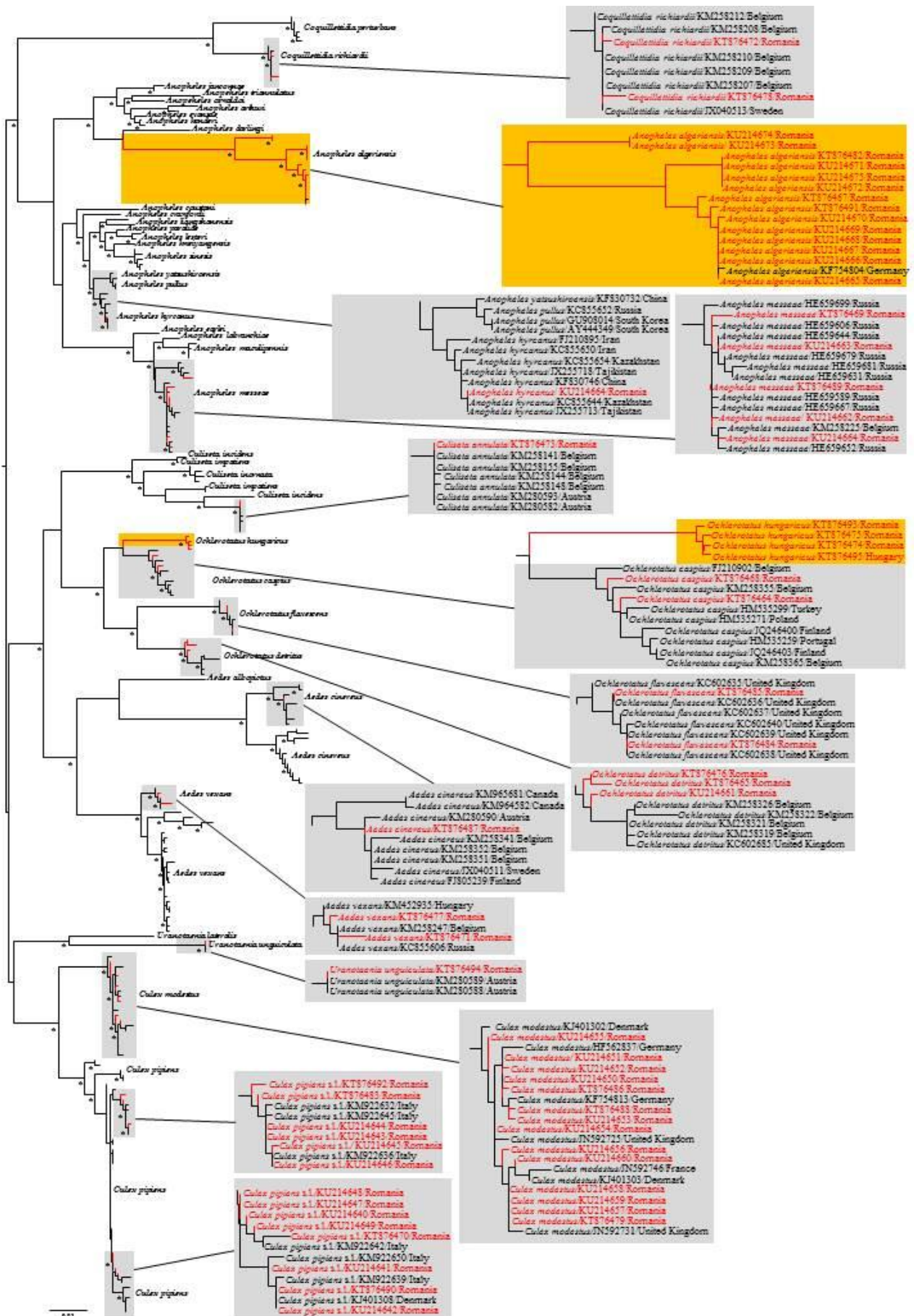
Figure 3. Maximum-likelihood phylogenetic tree of Tamura-Nei distance model and neighbor-joining (NJ)
Ochlerotatus species has a complex structure incorporating *Oc. hungaricus* as sister of O*c.*

caspius. Detailed relationships beetwen species gropus collected in the DDBR area is presented in figure 3.

4.3 Ecology

The number of species identified by us in the DDBR in the two years of investigations is high in comparison with the literature data. The number of detected taxa per sampling sites and calendar weeks varied between five to fourteen, with the lowest number of taxa in the sampling material from April and highest number of detected taxa in June. The highest numbers of mosquito specimens per calendar week were collected at the beginning of June, followed by two peaks in the end of June and August. The investigated taxa showed different phenological patterns. For example: highest number of specimens of *Ae. vexans* and *Oc. caspius* were trapped early summer, while the most *Cx. modestus* individuals were sampled only in late summer. Another example is the number of observed generations, e.g. *Oc. caspius* produced a single population peak, while *Cq. richiardii* and *An. hyrcanus* have three, respectively two population peaks, in correlation with the highest temperature values and abundant rainfall in the area investigated by us. The mosquito populations over the sampling period were dominated by taxa, which lay their eggs in the water, whereas taxa laying their eggs on the soil were only present in the beginning of the sampling period. The overwintering stages of the taxa followed have the highest proportion of species overwintering in the egg stage at the beginning of the year, followed by taxa overwintering in the larval stage, and were finally dominated by taxa, which overwinter as females. Univoltine taxa had their highest proportion during the summer month, whereas multivoltine species were present during the whole sampling period.

The mosquito fauna of the trapping sites was clearly dominated by two species: *Cq. richiardii* and *An. hyrcanus*. For Romania, both were previously reported to have their main distribution in the DDBR and surrounding floodplains (Nicolescu et al. 2003b). *Coquillettidia richiardii* has a specialized live cycle with larvae and pupae living permanently submerged and obtaining oxygen from the aerenchyma of various aquatic plants in permanent water bodies, finding perfect conditions in the DDBR. Similar breeding site preferences for stagnant water bodies with rich aquatic vegetation were described for *An. hyrcanus*. Both species are multivoltine (Gutsevich et al. 1974, Becker et al. 2010) and had two (*An. hyrcanus*) respectively three populations peaks (*Cq. richiardii*) during the study year. Due to their dominance, representing over three quarter of all collected specimens, the overall phenology and temporal pattern of functional groups basically followed the pattern of both species with three distinct population peaks, domination of the oviposition site "water" (both species) and domination of the overwintering stage "larvae" (*Cq. richiardii*) and "female" (*An. hyrcanus*).

We used MANOVA pairwise statistical test for our comparative analyses between collection sites. The results are presented in table 3., showed the p-value.

Table 3. Pairwise MANOVA analyses between collection sites, 2014-2015 and Total area. Bold numbers showed the significant values p ≤ 0.05.

2014-period	DUNAREA VECHE	LAKE ROSULET	LETEA	SULINA
DUNAREA VECHE		0.33719	0.6261	0.76293
LAKE ROSULET	0.33719		0.075	**0.0223***

	DUNAREA VECHE	LAKE ROSULET	LETEA	SULINA
LETEA	0.6261	0.075		0.07728
SULINA	0.76293	**0.0223**	0.07728	
2015-period	DUNAREA VECHE	LAKE ROSULET	LETEA	SULINA
DUNAREA VECHE		**0.00503**	**0.00358**	**1.20E-08**
LAKE ROSULET	**0.00503**		**0.00366**	**2.70E-07**
LETEA	**0.00358**	**0.00366**		**9.88E-07**
SULINA	**1.20E-08**	**2.70E-07**	9.88E-07	
Total (2 years)	DUNAREA VECHE	LAKE ROSULET	LETEA	SULINA
DUNAREA VECHE		0.54678	0.2241	**0.00881**
LAKE ROSULET	0.54678		**0.00891**	**9.30E-05**
LETEA	0.2241	**0.00891**		**0.0008**
SULINA	**0.00881**	**9.30E-05**	**0.0008**	

Principal Component Analysis (PCA) was used in four different habitats and our results demonstrate that the difference in community composition in time were not significant. PCA results show that the habitats are not well separated. Only the Lake Roșuleț ecosystem are separated from the others base on the massive presence of two species: *Cq richiardii* and *An. hyrcanus* (Figure 4.).
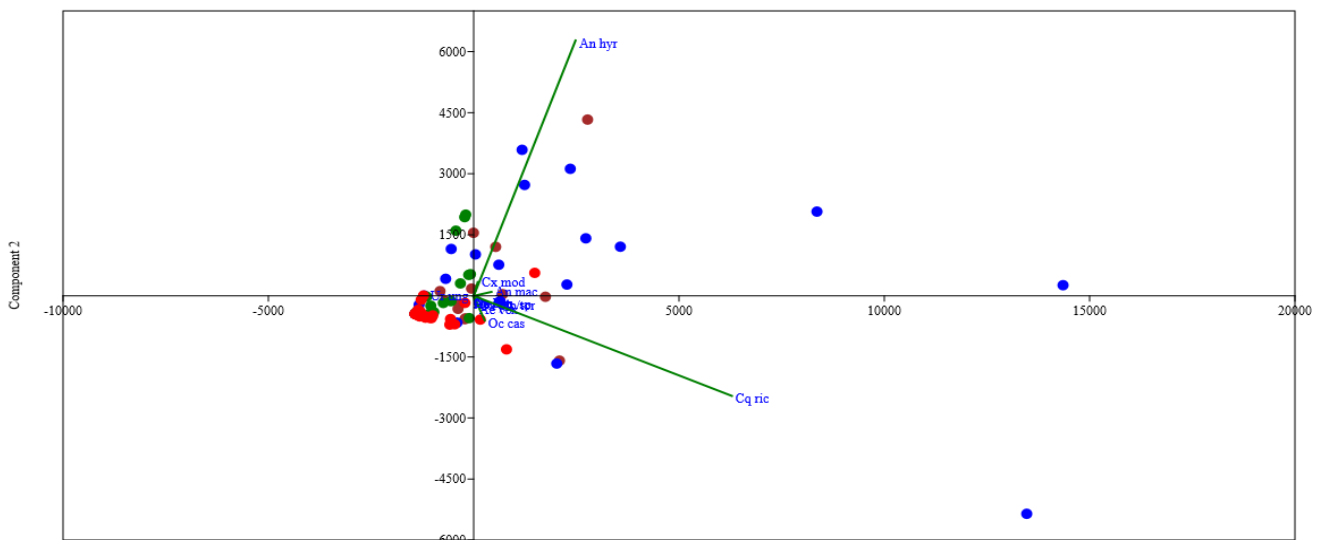

Figure 4. Principal Component Analysis between four study areas: blue- Lake Roșuleț, brown- Dunărea Veche, green- Letea, red- Sulina

Discriminant analysis (DA) was used to test the separation of the four different habitats investigated. DA maximized differentiation among groups, but not separate clearly but the most different habitat proved to be the urban area in Sulina, based upon species community and sampling times. The remaining study areas, Lake Roșuleț, Dunărea Veche and Letea are more closely related (Figure 5.). The results showed that the urban area has the most peculiar Culicidae fauna, different from the natural ones, because of the high number of "human produced" breeding sites, such as technotelmas.
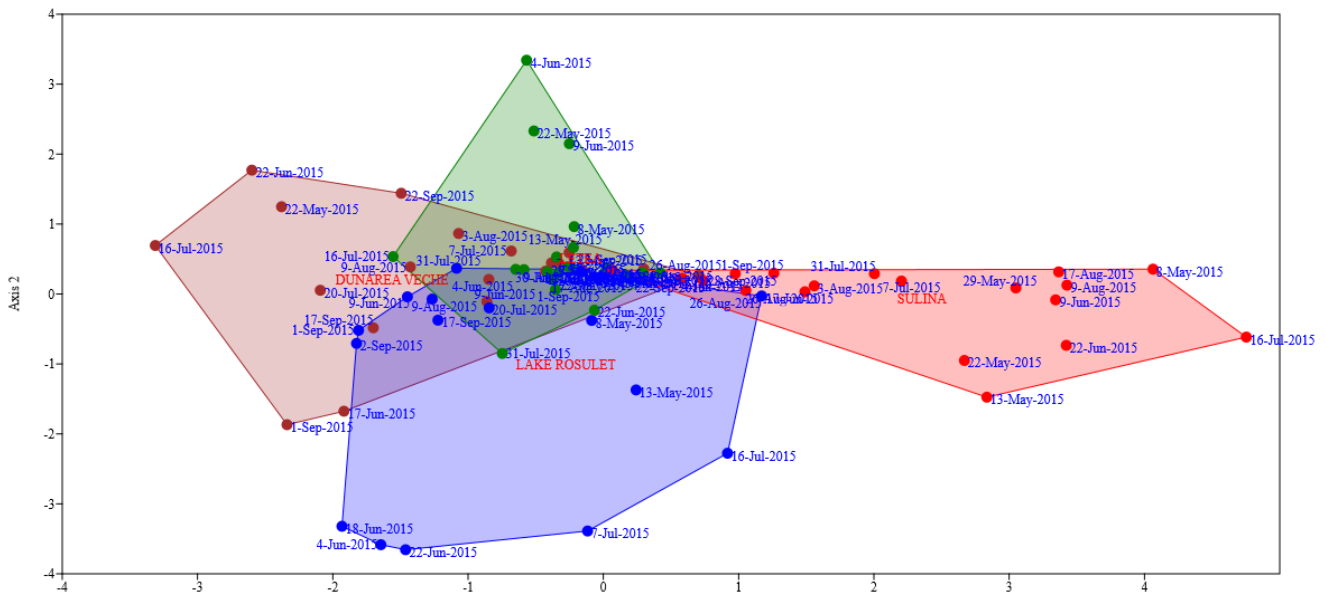
Figure 5. Discriminant analysis between four study area: blue- Lake Roșuleț, brown- Dunărea Veche, green- Letea, red- Sulina

Hierarchical cluster analysis has significant bootstrap support separated Lake Roșuleț (100 AV/BP value %) from all others. Dunărea Veche is also well separated (AV=99/BP=90) from Letea and Sulina. The dendrogram represented Letea and Sulina are near but not well supported (AV=67/BP=40) (Figure 6.).
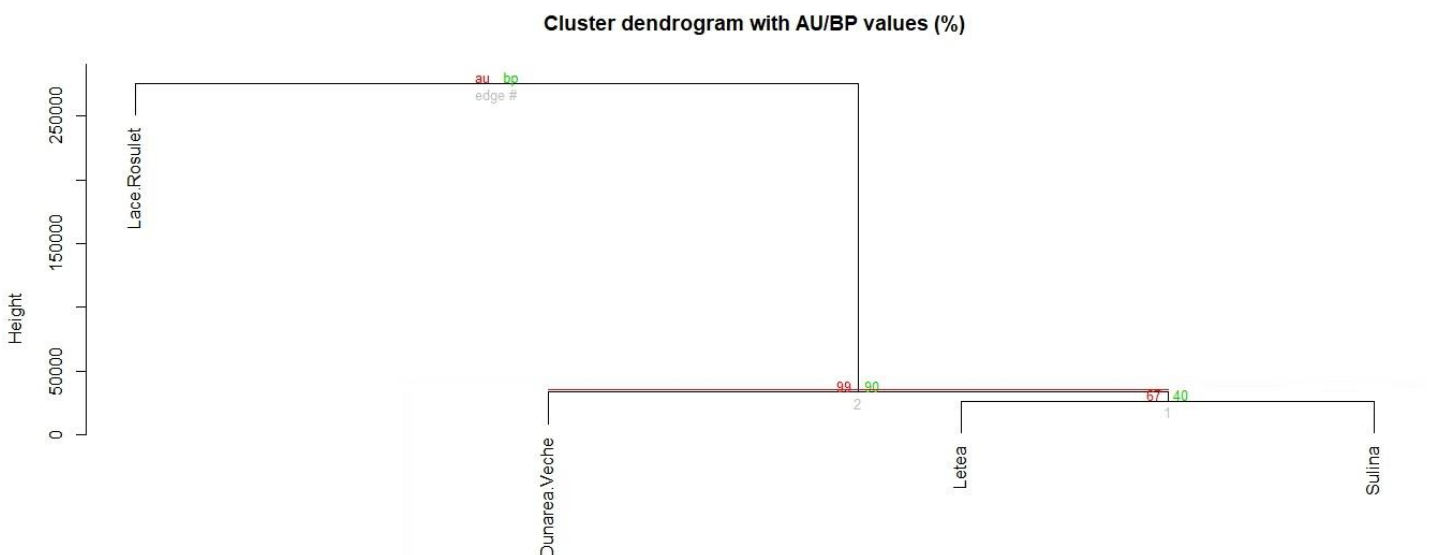

Figure 6. Dendrogram resulting from the Hierarchical cluster analysis with AU/BP bootstrap values (Distance is euclidean and cluster method is ward)

The highest diversity collection period is May and the second is June (Table 4.) with the most diverse collection sites the Letea forest ecosystem and Sulina urban ecosystem (Table

15

5.). The highest numbers of mosquito specimens were from Lake Roșuleț, an aquatic ecosystem.

Table 4. Summering information of collection amounts for Biodiversity analyses.

	April	May	June	July	August	September
Taxa	5	14	14	13	9	9
Individuals	30	15106	234857	207581	53668	22934
Dominance	0.4844	0.2393	**0.603**	0.4581	0.374	0.3693
Simpson	0.5156	**0.7607**	0.397	0.5419	0.626	0.6307
Shannon	1.026	**1.772**	0.9107	1.101	1.214	1.326
Evenness	**0.5579**	**0.4202**	0.1776	0.2314	0.3742	0.4185
Fisher alpha	**1.713**	**1.521**	1.145	1.067	0.8108	0.8856
Richness	6	14	14	19	9	9

Table 5. Summering information of collection sites for Biodiversity analyses.

	Dunarea Veche	Lace Rosulet	Letea	Sulina
Taxa	13	11	15	15
Individuals	102473	311046	51507	70074
Dominance	0.3517	**0.5671**	0.2907	0.3748
Simpson	0.6483	0.4329	**0.7093**	**0.6252**
Shannon	1.331	0.8878	**1.462**	**1.432**
Evenness	**0.2913**	0.2209	0.2875	0.2791
Fisher_alpha	1.14	0.8594	**1.43**	1.385
Richness	13	11	16	16

Regarding the ecology of mosquito species with human importance 6 species: *An. maculipennis s.l.*, *Ae. vexans*, *Cx. modestus*, *Cx. pipiens s.l.*, *Oc. caspius*, *Cq. richiardii*, a relative high number of individuals were detected early in May but the most abundant populations were identified by us in month June and July which is also a period with importance human concentration in the area (Figure 7.). All this species are commune in the
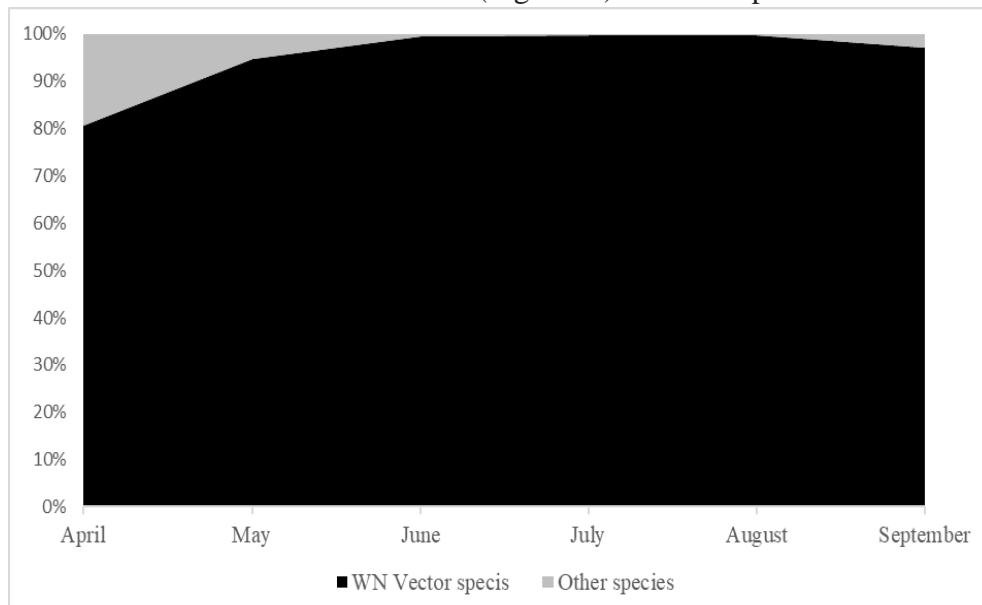

Figure 7. Summary of collection focus on vector species and percentage distribution

area, their activity correlate positive with high temperature (around 20 °C, exceptions Culex species with high activity at 24-26 °C) and high level of humidity in the area.

Detailed discussion of all 6 collected vector species were presented as follows:

1. ***Anopheles maculipennis messeae* (Falleroni, 1926)**

It is a widespread species present in northern and central Europe. It is also a common species in Romanian. This species belongs to the *Anopheles maculipennis* complex which is a classic example of a "species complex", containing at least 8 different species. Morphological characters of adults and larvae are highly similar, only the eggs are different. Our result of niche modelling of this species in the DDBR area revealed it is generally distributed along all channels of the Danube. The model uses three components, explaining 39,9% of the total distribution model: vegetation productivity index (VPI_2016apr), riverine and fen scrubs (eco_f38), low density buildings (eco_f55), arable land and market gardens index (eco52) (Figure 8.).

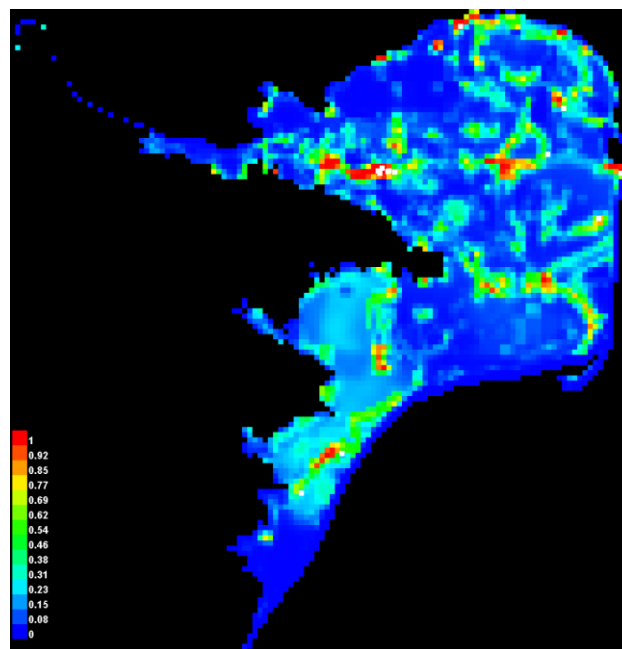This species has strong populations from in the Dunărea Veche and Lake Roșuleț ecosystems

Figure 8. Result of Niche model, *An. maculipennis messeae* distribution at Danube-Delta

based on the evidence of our collected specimens. Populations can survive winter weather. It was collected large numbers during the first year, in June and July. It has two or more generations per year. Results of MCMCglmm model found significant positive correlation between temperature and abundance of the species on the collection day and also one week and one month earlier before our investigation, but between any wind one week before has significant negative correlation the emergence period. The model showed that the species has a single population peak.

I found a significant correlation between high temperature and low abundance on the day of the collection, so high temperatures are not good for species activity. The high mean temperature one- or two weeks before the emergence period influenced negatively the abundance of species. In contrast the high mean precipitation value positively influenced the

development of larvae. On the collection day temperature parameters required for imago activity was around 20-26°C, they prefers no- or little precipitation, showing an increasing trend of abundance at 4-8 m/s wind strength and high humidity (Figure 9.).

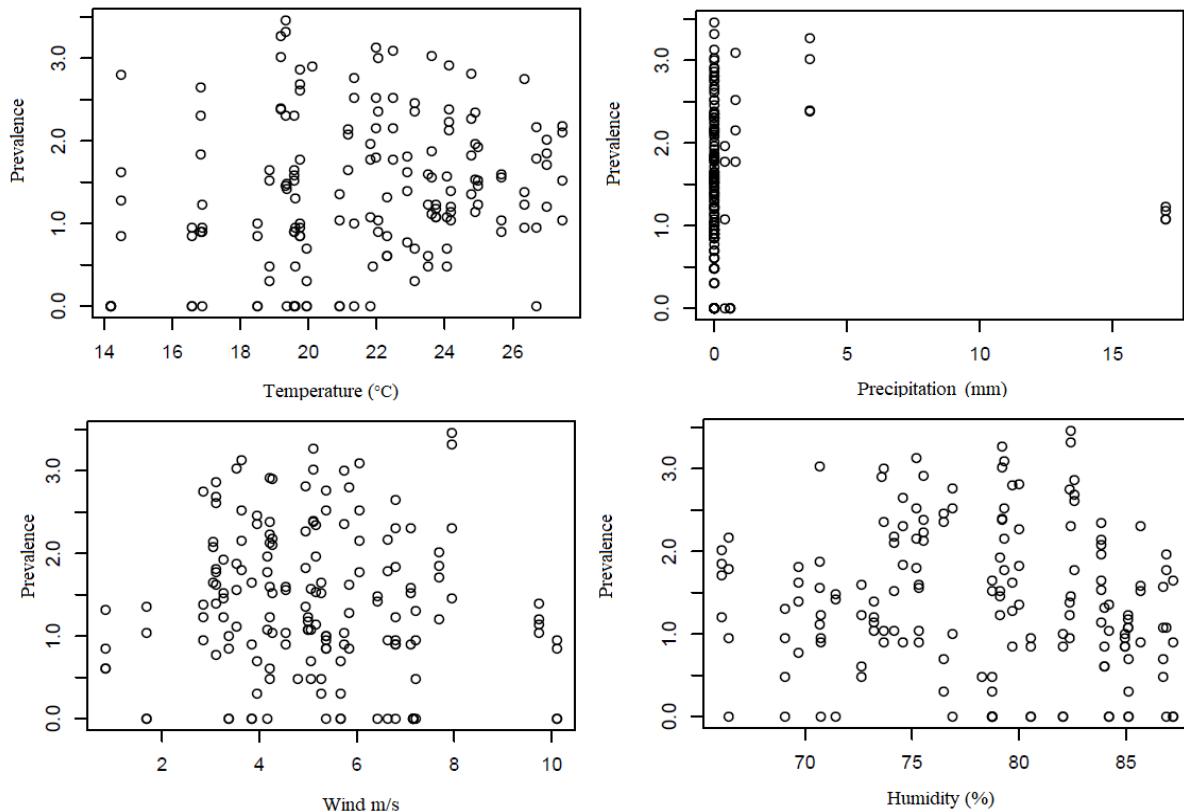The females are essentially zoophilic feeding almost exclusively on domestic animals;

Figure 9. Temperature parameters required for *An. maculipennis* activity using to data day of collection by MCMCglmm model generated

thus contact also humans. Blood meals are taken from humans only when the density is very high and there is a shortage of livestock, but they may also attack humans in houses (Barber & Rice 1935, Becker et al. 2010). Described the species as endophilic as it was found resting during the daytime in human buildings (stables and cellars too) (Becker et al. 2010). It is the most important vector of malaria transmission, and can potencionaly transmit Batai virus in Europe, with a veterinary importance, having lethal effect on rodents. The species is also host for the bacterium *Francisella tularensis*, the causative agent of tularemia, isolated only from natural populations (Detinova & Smelova 1973).

Our data has shown that the species has two peaks in the flight period, in June and again in July, suggesting two generations per year.

2. *Aedes vexans* (Meigen, 1830)

This species has a worldwide distribution including all European countries. *Ae. vexans* prefers breeding in inundated areas such as floodplains of rivers or lakes with fluctuating water levels, revealed also by our niche modelling results (Figure 10.). The model can be used with three components, explaining 66,7% of the total distribution model: low density buildings (eco_f55), vegetation productivity index (VPI_2016apr), 1km distance to water
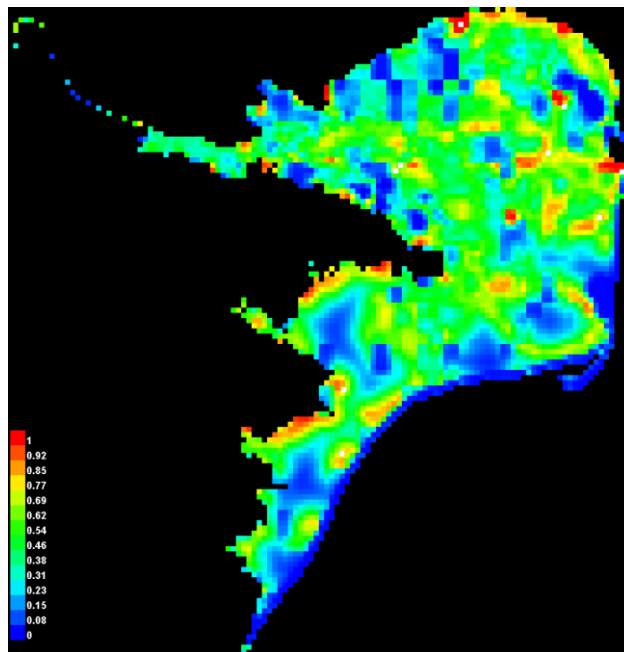
Figure 10. Result of Niche model, *Ae. vexans* distribution at Danube-Delta

(dist2water). In the period of our collection the species has strong populations with high number of specimens in the Danube Delta area. Results of MCMCglmm model found a negative significant correlation between the high abundance of the mean wind and humidity a week prior to emergence. In the days of our collections the meteorology parameters required for imago activity were 20-24°C temperature, with no precipitation, and a wind strength of 3-6 m/s as well as increased humidity (Figure 11.).
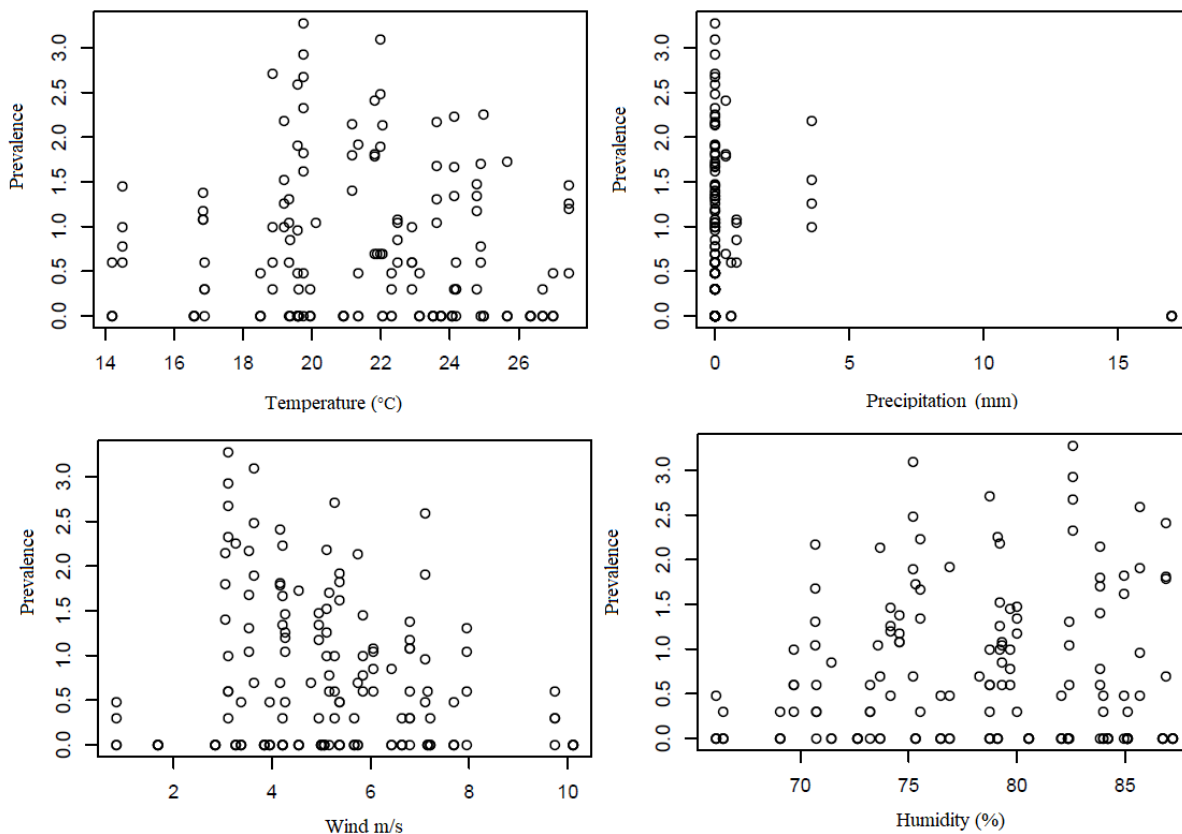

Figure 11. Temperature parameters required for *Ae. vexan*a ctivity using to data day of collection by MCMCglmm model generated

Ae. vexans prefers forest habitats, having multiple generations per year. This was confirmed by our results which detected two peaks in the flight period. In 2014 the species was active in June and July, but the highest peak was in May in 2015. Based on our observation as well as on literature data we can conclude that only a part of the population returns to their original breeding sites after a blood meal, while a considerable part of the population migrate and lay their eggs far away from their original breeding sites.

Ae. vexans it is considered an ideal bridge vector species. It is widely distributed, can become very abundant, often at the same time when virus activity is at its peak, it feeds readily on humans and domestic animals, and it has been found naturally infected with various moboviruses (Reinert 1973, Molaei & Andreadis 2006). In Europe, *Ae. vexans* is involved in the transmission of Tahyna virus (Hubálek 2008, Becker et al. 2010), West Nile virus (Kenyeres & Tóth 2008) and the bacterium *Francisella tularensis*, the causative agent of tularemia which were isolated from the mosquitoes natural populations (Detinova & Smelova 1973). Additionally, they can transmit *Dirofilaria repens*, as it was demonstrated by Ionică et al. 2017 in an investigation on Romanian mosquito populations.

3. *Culex modestus* **Ficalbi, 1890**

Cx. modestus is a widely distributed species in Europe, and it is a common species in the southern and central countries too. The larvae show a preference for shallow sunlit habitats and are frequently found on meadows, in irrigation channels, inundation areas of rivers, or rice fields. Other common breeding waters are pools, ponds, swamps, and marshes with rich vegetation. In breeding situations the water may be fresh or slightly saline. We collected large number from all our collection sites. Results of niche modelling is show in figure 12. It prefers sedges, reeds, bulrush and marshes. The model can be used with three components, explaining 46,5% of the total distribution model: Romanian population 2011 scale, soil: clay and pH
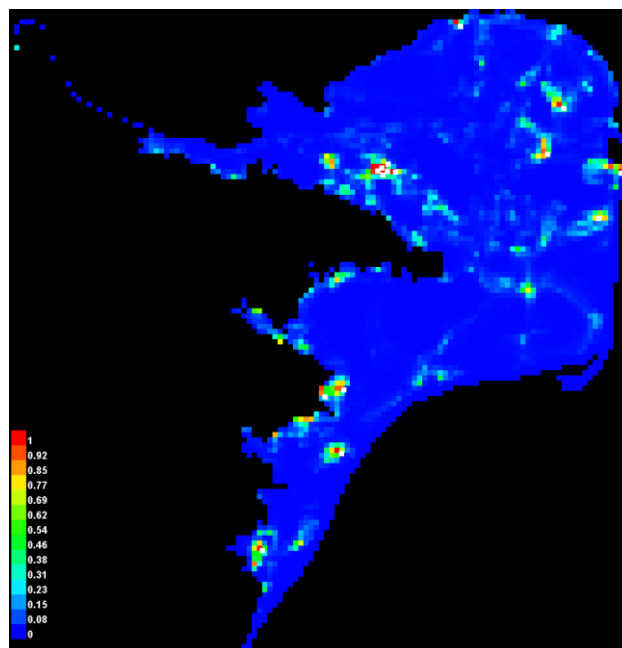

Figure 12. Result of Niche model, *Cx. modestus* distribution at Danube-Delta

content. Distribution is segregated in DDBR, the species was detected only from a few habitats. Results of MCMCglmm model found significant correlation between precipitation, humidity and abundance in collecting period from two weeks and one month earlier. The model showed positive correlation between abundance and high temperature. On the collection days the meteorological parameters required for imago activity were 20-26°C temperatures and it preferred no precipitation, but was not influenced by wind or humidity (Figure 13.).

The flight period in 2014 is shown as having a growing trend of the abundance of the
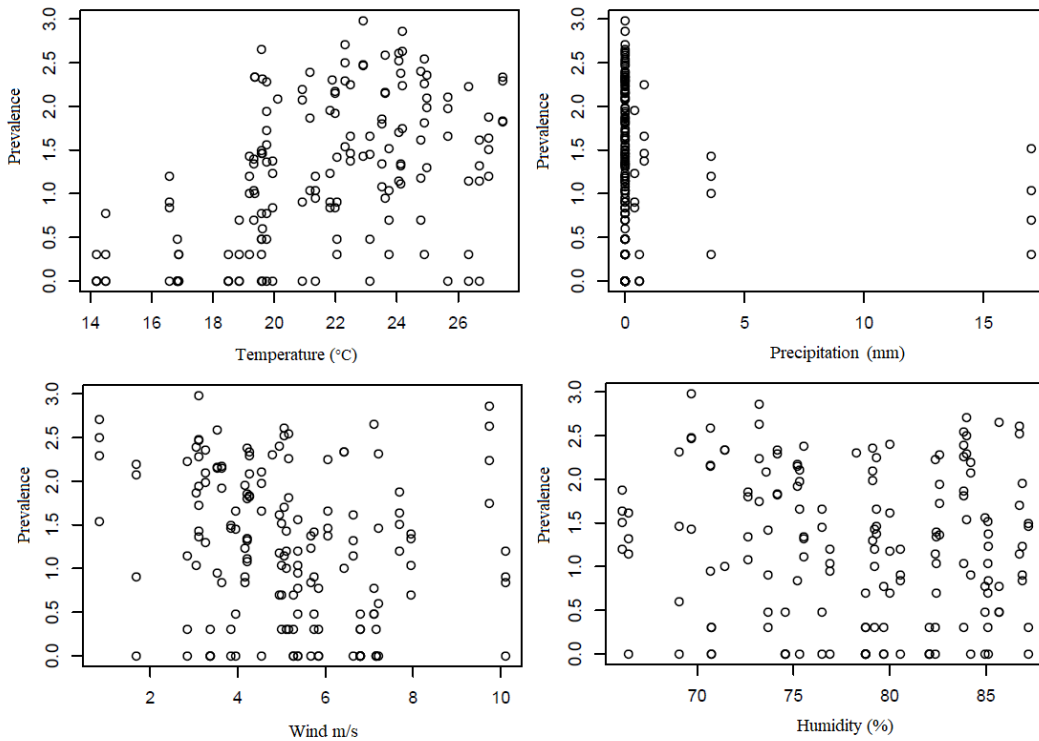

Figure 13. Temperature parameters required for *Cx. modestus* activity using to data day of collection by MCMCglmm model generated

species to the end of the vegetation period, but in 2015 has two peaks in July and September. Among the *Culex* genus there were two frequent species, *Cx. modestus* and *Cx. pipiens*. The flight period of *Cx. modestus* observed in two consequent years show similar patterns, having two peaks, a small one at the beginning of July, and the second, higher peak at the beginning of September, with a slightly higher number of individuals in 2014 than in 2015.

Cx. modestus females proved to be really aggressive to humans, preferring urban ecosystems and were active all day long with feeding preferences for mammals. However, this species not travel far from the larval breeding sites, being characteristic close to human settlements. It is a vector species, has repeatedly been reported as an mobovirus vector of two different Bunyaviremia, Tahyna and Lednice (Lundström 1994) and is also regarded as a potential vector of West Nile virus (Golding et al. 2012). In addition, *Francisella tularensis* has been found in naturally mosquito populations transmitting tularemia (Gutsevich et al. 1974). Between 2011 and 2013, different mosquito species in Romania have been tested positive for West Nile virus (Dinu et al. 2015). *Cx. modestus* is considered to be the main vector

species of West Nile virus in Europe (Balenghien et al. 2008, Nicolescu 1998, Golding et al. 2012, Dinu et al. 2015).

4. *Culex pipiens/ torrentium* **Linnaeus 1758**

This species is widespread in the Holarctic region and found throughout Europe, it is also a common species in Romania. It is able to inhabit nearly every kind of water source. The
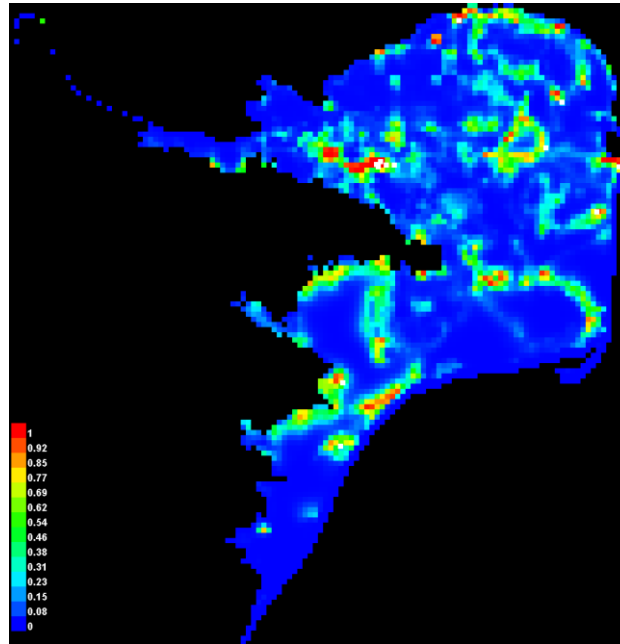

Figure 14. Result of Niche model, *Culex pipiens/ torrentium* distribution at Danube-Delta

larvae frequently occur in manmade water bodies such as flooded cellars, construction sites,
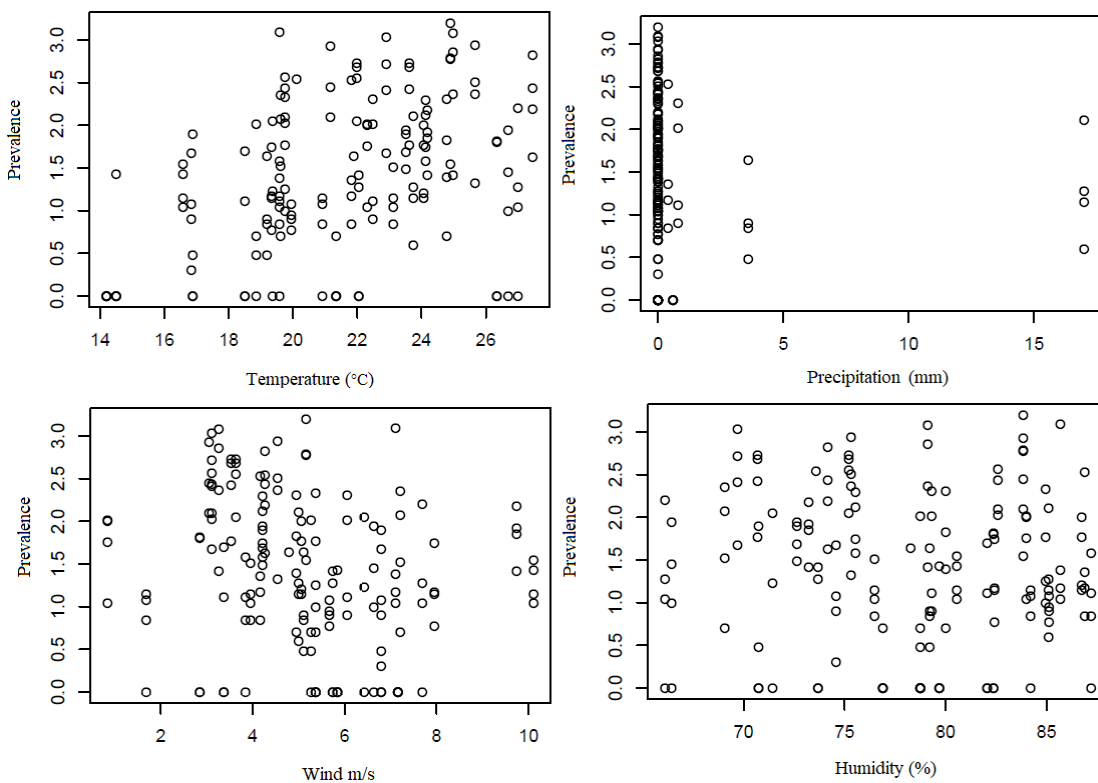

Figure 15. Temperature parameters required for *Cx. pipiens/ torrentium* activity using to data day of collection by MCMCglmm model generated

water barrels and tin cans, metal tanks, ornamental ponds, and containers in gardens and in churchyards. They even tolerate a small amount of salinity and can occur in rock pools. We collected this species in large numbers from all collection sites. Results of niche modelling are shown in figure 14.

The species has a wide range of habitat preferences. The model can be used three components, explaining 37,8% of the total distribution model: water distance (dist2water1km_scale), arable land and market gardens (eco_f52), riverine and fen scrubs (eco_f38). The distribution is large in DDBR. Results of MCMCglmm model found positive significant correlation between temperature, precipitation, humidity, wind and abundance from one month earlier to our sampling. The model demonstrated a significant correlation between high temperature, precipitation and abundance the one week and two weeks earlier before our sampling. High humidity and wind from one month earlier shows a positive influence on the abundance of the adults. On the day of collection the meteorological parameters required for imago activity are 20-26°C temperatures, it prefers no precipitation, but is not influenced by wind or humidity (Figure 15.).

The species can develop several generations per year depending on climatic conditions. This pattern was also supported by our results, having three or more peaks in the flight period.

Cx. pipiens provided to be the most troublesome mosquitoes species, biting (feeding), almost exclusively at night, and females feeding only on birds (Farajollahi et al. 2011). However, Börstler et al. in 2016 tested feeding preference of *Cx. pipiens* and the results were surprising since they feed not only on birds but on mammals and humans too. It occurs frequently and in a high number in houses and in practically all types of human and animal shelters in urban ecosystems. In such "human made" environments this species has multiple generations rather few in natural ecosystems, which is also reflected by the flight period *Cx. p. pipiens* seems to play a minor role as an mobovirus vector in Europe (Farajollahi et al. 2011). Nevertheless, one report of high incidence of WNV from Romania (Nicolescu 1998) and a record of low incidence of Ockelbo virus from wild caught females (Lundström 1994) demonstrate the capacity of the species for moboviruses. Additionally, they can transmitt *Dirofilaria repens* and *D. immitis* nematodes were detected in Europe.

5. *Ochlerotatus caspius (Pallas, 1771)*
Oc. caspius is a Holarctic species, widely distributed in Europe too. It has a high tolerance to salt and dryness. It is regarded as a seaside mosquito that readily breeds in inland salt marshes and freshwaters with 0.5 g NaCl/l (Becker et al. 2010). We collected this species a large number from all collection sites. Result of niche modelling is show in figure 16., the species preferred irrigated perimeter, water courses. The model used three components, explaining 73,4% of the total distribution model: vegetation condition index (VCI_2016apr_scale), soil clay scale (eco_f38), riverine and fen scrubs (eco_f52). Our model forecast a widespread distribution in DDBR. Results of MCMCglmm model found significant correlation between abundance and temperature, precipitation, humidity and wind in the sampling days, but also from one week and one month prior to emergence. The model showed that there is a significant correlation between mean temperature, precipitation and humidity to abundance on the day of collection, so the high temperature, precipitation and high humidity is

not good for the species activity. The abundance was positively influenced by the mean temperature from one week before prior to collection. The high temperature from one month before collection has a positive influence on the abundance. In the days of sampling the

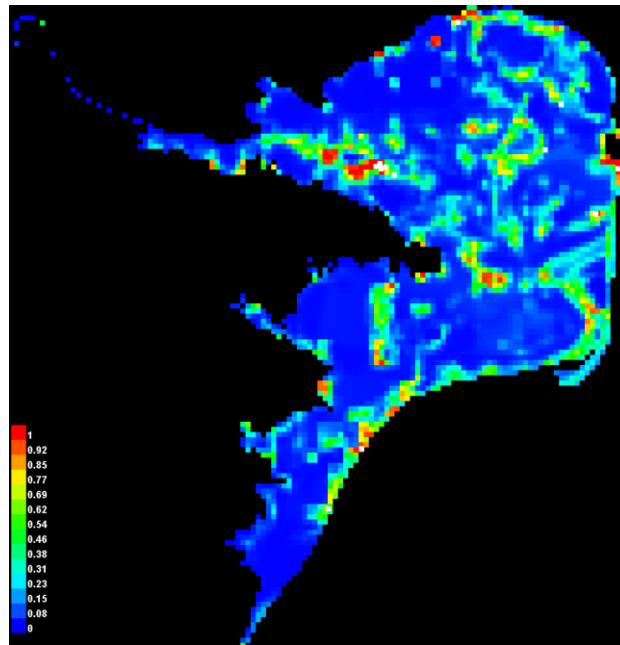Figure 16. Result of Niche model, *Oc. caspius* distribution at Danube-Delta

meteorological parameters required for imago activity were 20-22°C temperatures, it prefers no precipitation, wind strength at about 3-6 m/s has a positive influence as well as the increasing humidity (Figure 17.).
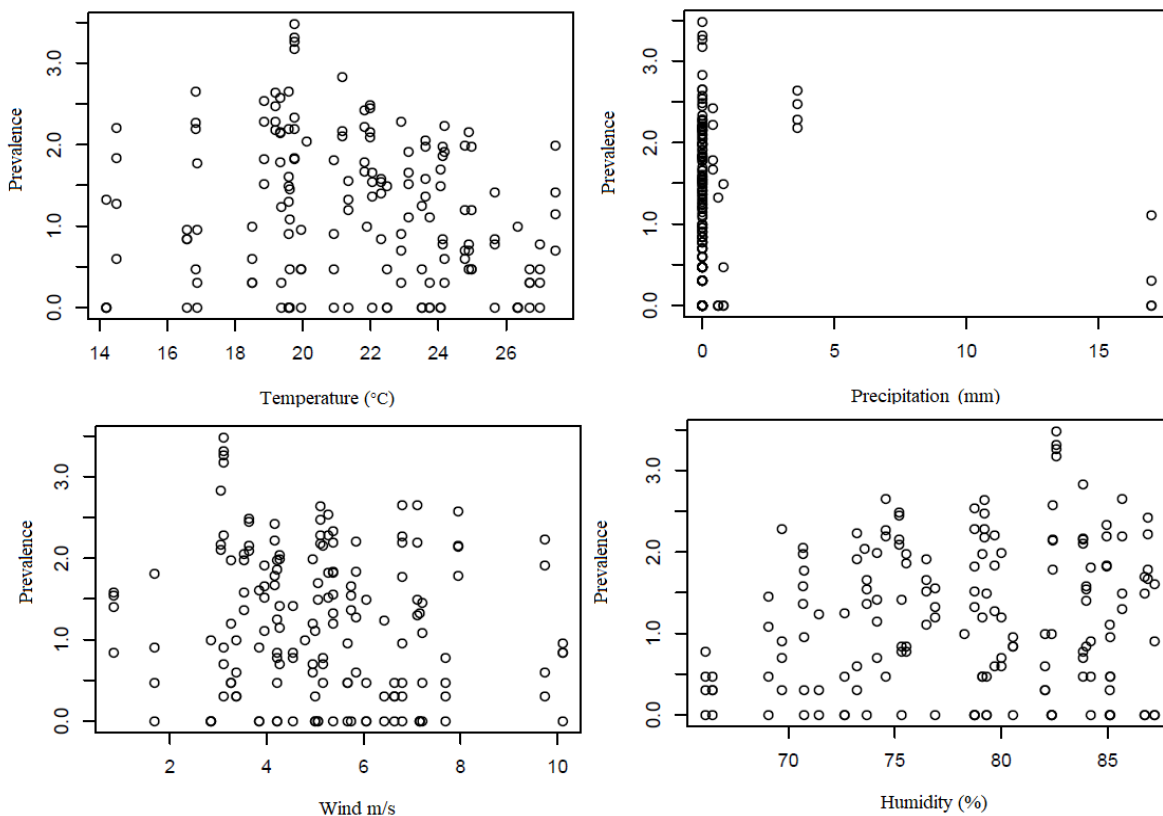

Figure 17. Temperature parameters required for *Oc. caspius* ctivity using to data day of collection by MCMCglmm model generated

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Oc. caspius is a polycyclic, halophylic species, also shown by our data. The species has only one generation per year sometimes (like in 2014), but sometimes has multiple generations (like in 2015) depending on the nature of the breeding site. The females bites humans and mammals (wild and domestic animals) (Kenyeres & Tóth 2008). They often bite during the day and night, but usually most actively search for a blood meal at dusk. The species has a high resistance to heat and drought. Females actively search for blood at temperatures ranging from 11.5 to 36°C and relative humidity ranging from 47 to 92% (Petrić 1989). They may migrate for long distances, up to 10 km (Becker et al. 2010).

It is a bridge vector species, potential transmitting viruses like Tahyna virus and West Nile virus (Medlock et al. 2005) and nematodes such as *Dirofilaria repens* and *D. immitis*, but additionally spreading bacterium like *Francisella tularensis*, the causative agent of tularemia, could be detected in natural populations (Detinova & Smelova 1973). These mosquitoes could possibly infected and transmit rabbit myxoma viruses too (Kenyeres & Tóth 2008). In Romania it was detected positive for West Nile virus.

6. *Coquillettidia richiardii (Ficalbi, 1889)*

Cq. richiardii is a common species throughout Europe, and it has been recorded from all countries. The mosquito fauna of the trapping sites was clearly dominated by *Cq. richiardii* species. For Romania, it was previously reported to have its main distribution in the DDBR and surrounding floodplains (Nicolescu et al. 2003b). *Cq. richiardii* has a specialized live cycle with larvae and pupae living permanently submerged and obtaining oxygen from the aerenchyma of various aquatic plants in permanent water bodies, finding perfect conditions in the DDBR. It is a multivoltine species (Gutsevich et al. 1974, Becker et al. 2010) and we also detected three population peaks during the study years. Individuals of this species counted for
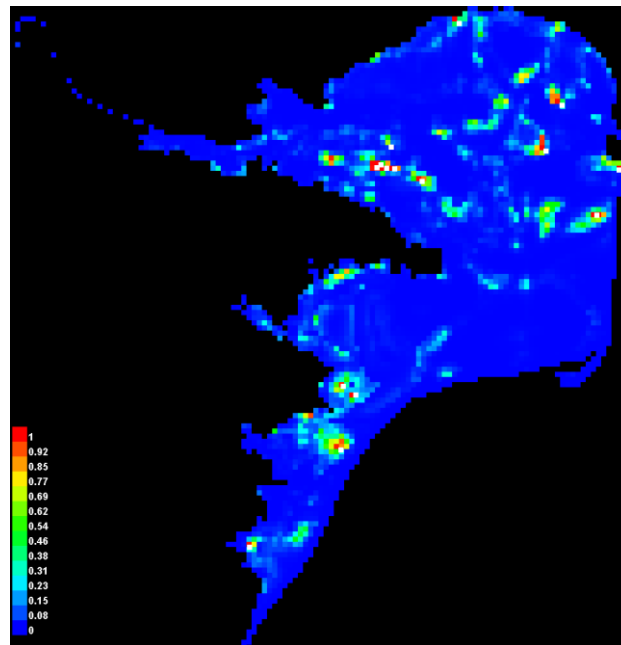

Figure 18. Result of Niche model, *Cq. richiardii* distribution at Danube-Delta

over three quarter of all other mosquitoes collected.

The overall phenology and temporal pattern of functional groups basically followed the pattern of species with three distinct population peaks, domination of the oviposition site "water" and domination of the overwintering stage "larvae". The species was most abundant at the Lake Roșuleț sampling site. Result of niche modelling is show in figure 18. The species prefer lake and water courses. The model used three components, explaining 45,1% of the total distribution model: Romanian population in 2011 scale, soil silt scale and fen scrubs riversides areas (eco_f38_scale). In the sampling period the species has strong populations in DDBR.

Results of MCMCglmm model found significant correlation between abundance and temperature on the sampling day. We found a significant correlation between high temperature and low abundance on the day of collection, so the high temperature is not good for the species
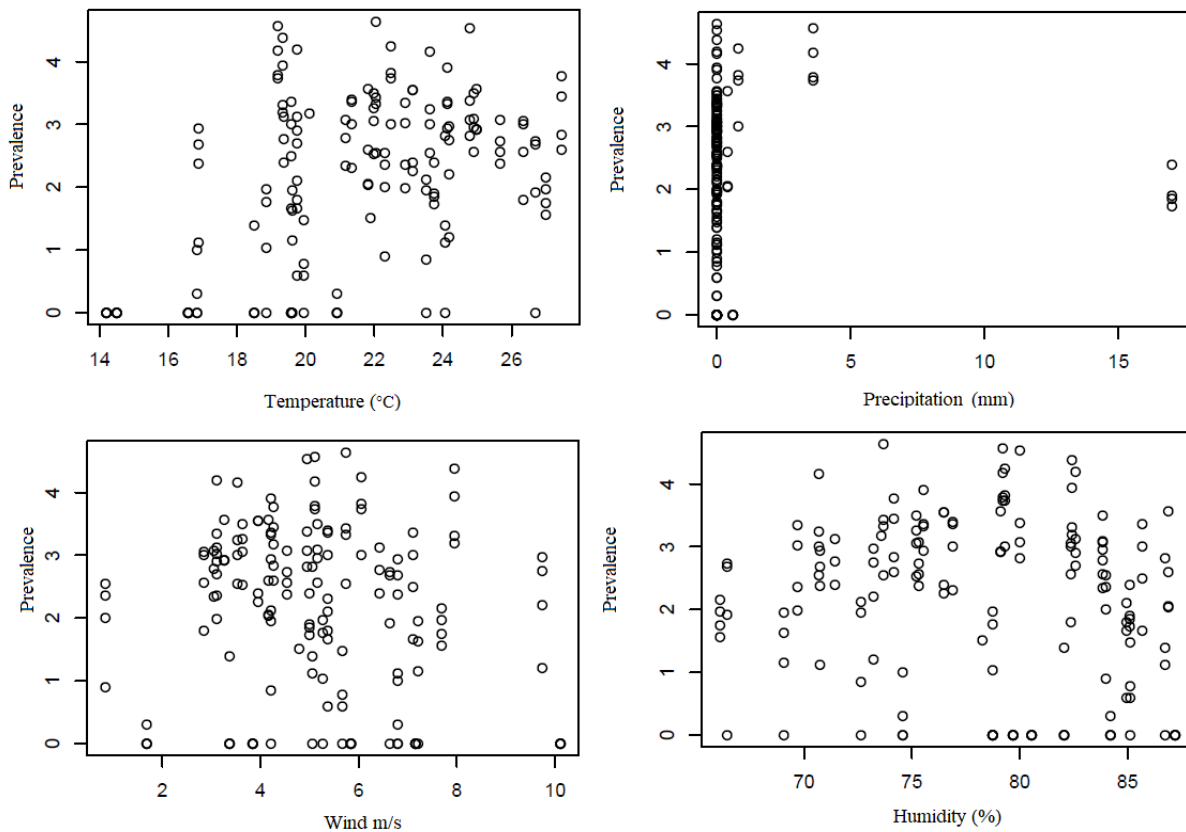
Figure 19. Temperature parameters required for *Cq. richiardii* ctivity using to data day of collection by MCMCglmm model generated

activity. The mean temperature and precipitation from one week before collections has a positive influence on abundance. The mean temperature from two weeks and one month before collections has a positive influence on abundance. From a month and two weeks before collecting, there was a negative significant correlation between the mean wind, humidity and abundance of the species. In the day of sampling meteorological parameters required for imago activity were around 20-26°C temperatures, preferring low precipitation, but the abundance is positively correlated with wind strength 4-8 m/s and increasing humidity (Figure 19.).

Based upon literature data *Cq. richiardii* has only one generation per year in the North (Service 1969) and 2-3 generations in the South of Europe (Gutsevich et al. 1974). Our results also show two generations in both of the two years investigated by us.

The species has also been reported as autogenously, but some females may be unable to develop their first eggs without taking first a blood meal. Biting activity was recorded at a temperature between 9 and 26°C and a relative humidity between 30 and 92%. Feeding preference are mammals (Becker et al. 2010) but may also take their blood meal from birds (Service 1969) and amphibians and humans too (Kenyeres & Tóth 2008).

In Romania, WNV was also detected in mosquito pools of the species *Cq. richiardii* (Nicolescu 1998) highlighted that this species might play an important role in the transmission cycle of WNV, if the principal vector species are missing or present only with low densities. The mosquitoes can be potential bridge vector to transmit WNV (Medlock et al. 2005), Tahyna and Batai viruses which causes illnesses in animals.

4.4   Parasitology

Potentially dangerous mosquito species (which feed preferentially on human blood) are also disease-causing vectors for humans and 6 vector species were identified in the DDBR area from this category. These were also present during the entire sampling period, accounting for more than 75 % of the total number of collected specimens and exceeding 50 % of all collected specimens for most calendars weeks in the summer.

4.4.1. Mosquito-borne viruses

Mosquito-borne viruses DDBR

From both collecting years a total of 0.5% of mosquitoes showed as virus infected. Members from the family of Flaviviridae viruses were detected in *An. hyrcanus* and *Oc. caspius* species (from Sulina, Lake Roșuleț and Dunărea Veche collection sites) Members of the family of Rhabdoviridae viruses found by us offer new data to the region. Rhabdoviruses were detected in *Oc. caspius*, *Oc. dorsalis*, *Ae. vexans, Oc. detritus* and *Cq. richiardii* (from Lack Roșuleț, Sulina Letea, Dunărea Veche collection sites).

Between 2011 and 2013, different mosquito species in Romania have been tested WNV positive (Dinu et al. 2015). *Culex pipiens* s.l. is considered to be the most important WNV vector in the country (Nicolescu 1998, Dinu et al. 2015), and together with *Cx. modestus* are considered to be the main vector species of WNV in Europe (Savage et al. 1999, Balenghien et al. 2008). However, in Romania, WNV was also detected in mosquito pools of the species *Cq. richiardii*, *An. hyrcanus*, *Ur. unguiculata*, *Oc. caspius*, and *An. maculipennis* s.l. (Nicolescu 1998) highlighted that these species might play an important role in the transmission cycle of WNV, if the principal vector species are missing or present only with low densities. During the entire sampling period, a huge proportion of the mosquito population can be classified as potential WNV vectors. With 40 % of all collected specimens, the most frequent species *Cq. richiardii* is probably also the most important vector of WNV in the DDBR, followed by *Cx. pipiens* s.l./*Cx. torrentium*, *Oc. caspius*, and *Cx. modestus*, which were all found WNV positive in Romania (Dinu et al. 2015). *Anopheles hyrcanus* was the second most frequent species and also detected WNV positive in the country (Lvov et al. 2004, Dinu et al.

2015). However, due to the generally assumed host preference for mammals, the species probably do not play an important role as bridge vector.

Mosquito-borne viruses Cluj-Napoca

A high number of the Culicidae species (11 vector out of 14 species) identified by us in the Cluj-Napoca area represent a high potential risk for virus transmission, as they are already involved in virus transmission mechanisms in many other countries. The results of the mobovirus analysis were negative in the city, which suggests that the mosquito fauna from the area is not infected yet by any moboviruses. The present survey should serve as an early warning system for public health, particularly presenting a great interest in epidemiology by detecting possible new pathogens in the future and estimating their frequency in mosquitoes and the implications for humans as well.

During our work, we were not able to identify any moboviruses in the collected Culicidae material from Cluj-Napoca area, despite the presence of some suitable habitats hosting large and diverse mosquito communities here. There are a high number of important suitable wet habitats in Cluj-Napoca and its surroundings (lake ecosystems, the presence of the Someș River, forested boggy area), therefore the lack or the very low level of virus infection of the studied Culicidae community could be associated with the lack of possible hosts, like migratory birds or present only accidentally or unevenly.

4.4.2. Mosquito-borne nematodes

In 2014 3% of all mosquitoes collected (240 572 Culcidae specimens) were infected by parasitic nematodes. *Dirofilaria repens* and *D. immitis* were detected mostly from Lake Roșuleț in *An. hyrcanus, Cq. richiardii, Cx. pipiens, Cx. modestus, Ae. vexans, An. algeriensis, An. maculipennis.* Previously these were only detected in *Ae. vexans,* but during our investigation we were able to detect nematodes in 6 other species of mosquito too. Mosquito pools from 14 mosquito taxa were detected for *D. repens* and *D. immitis* DNA. From the 2,118 mosquito pools screened, 96 pools (4.53%) tested positive for *Dirofilaria* spp. DNA (EIR per 100 specimens = 0.041, 95% CI: 0.034–0.050), which further divided into 83 pools (3.92%) positive for *D. immitis* (EIR = 0.036, 95% CI: 0.029–0.044) and 23 pools (1.09%) positive for *D. repens* (EIR = 0.010, 95% CI: 0.006–0.014). In total, 10 pools (0.47%) were tested positive for both *Dirofilaria* species representing 10.42% of all pools tested positive for *Dirofilaria* spp. DNA. Between 2.85 and 5.49% of the tested mosquito pools per trapping site were positive for *Dirofilaria* spp. DNA. *D. immitis* was more prevalent than *D. repens* for all four sites (Tomazatos et al. in press).

Recent studies on *Dirofilaria* spp. in different Eastern European countries highlight significant local circulation with a wide range of mosquito species involved as vectors (e.g. Belarus or Moldova (Șulesco et al. 2016)). Our recent findings confirmed that in the DDBR area (Romania), mosquitoes should also present a high prevalence for *Dirofilaria* spp.. Further systematic xenomonitoring studies including different components of the *Dirofilaria* spp. transmission cycle (mosquito vectors, dogs as definitive and humans as secondary hosts) should be implemented in Eastern European countries to evaluate the local risk of human and

canine dirofilariasis, allowing the implementation of effective surveillance and control measures (Tomazatos et al. in press).

5.   CONCLUSIONS

Faunistic research

- We compiled an update checklist of the Culicidae fauna of Romania, containing in the present 60 mosquito species (10 more species than the previous checklist published by Nicolescu 1995)
- Here we published new faunistic data on 20 different Culicidae species, mostly from Transylvania
- We found a DNA barcode library, containing the standard DNA sequences of the Romanian Culicidae species (at now 314 sequence data from 19 different mosquitoes species)
- In the Danube Delta Biosphere Reserve area we collected more than half a million (538 617) of specimens belonging to 17 species and two new species to the Romania Culicidae fauna: *Anopheles algeriensis* Theobald, 1903 and *Ochlerotatus hungaricus* (Mihályi, 1955)
- 14 species (728 specimens) were identified by us in Cluj-Napoca, *Coquiuettidia richiardii* is reported first time from Transylvania

Taxonomy research

- 64 specimens were sequenced and generated a mtCOI phylogenetic tree
- Detected 3 species complex

Ecology research in DDBR

- We detected the highest diversity of Culicidae assemblages in the urban- and forest ecosystems, the urbanized environment has a different Culicidae fauna composition from those of natural habitats, which can be explain by the diversity of breeding habitats, missing from natural habitats (ex. artificial water collectors, like tires, recipes of different sizes) and the high number of potential food sources (humans)
- The highest number of specimens has been collected near the lotic ecosystems
- Collection period with highest diversity of species provided to be May and the second June
- 10 species identified by us in the present study could potentially be vector species, 6 of which are common species with high abundance in the DDBR and potentially dangerous for human
- All 6 vector species are activity correlate positive with high temperature (around 20 °C, exceptions Culex species with high activity at 24-26 °C) and high level of humidity in the DDBR.

Parasitology research

- In DDBR, in both collecting years (2014-2015) a total of 0.5% of mosquitoes showed to be virus infected. Members from the family of Flaviviridae viruses were detected in *An. hyrcanus* and *Oc. caspius* species (from Sulina, Lake Roșuleț and Dunărea Veche collection sites) Members of the family of Rhabdoviridae viruses found by us offer new data to the region. Rhabdoviruses were detected in *Oc. caspius*, *Oc. dorsalis*, *Ae. vexans, Oc. detritus* and *Cq. richiardii* (from Lack Roșuleț, Sulina Letea, Dunărea Veche collection sites)

- High number of the Culicidae species (11 vector out of 14 species) were identified by us in the Cluj-Napoca area, representing a high potential risk for virus transmission, as they are already involved in virus transmission mechanisms in many other countries. The results of the mobovirus analysis were negative in the city, which suggests that the mosquito fauna from the area is not infected yet by any moboviruses

- In 2014 3% of all mosquitoes collected by us (240 572 Culcidae specimens) were infected by parasitic nematodes. *Dirofilaria repens* and *D. immitis* were detected mostly from Lake Roșuleț in *An. hyrcanus, Cq. richiardii, Cx. pipiens, Cx. modestus, Ae. vexans, An. algeriensis, An. maculipennis*

Our results confirm that the areas investigated by us (the Danube Delta and surroundings of the metropolis Cluj-Napoca) need more comprehensive faunistic investigations in the future, because of the presence of potentially important vector species for a series of medically important pathogens. Both regions should be considered important biogeography crossroads of mosquitoes species and must be included in the European early warning system network and therefore more mosquito faunistic surveys are highly recommended from here.

Finally, this information can also help us to implement vector control programs depending on the life cycle of some mosquito species of medical importance and to adjust the timing of interventions, if necessary.

6. LIST OF PUBLICATIONS

The following articles were published, containing results from my PhD thesis:

Published

Török, E., Tomazatos, A., Cadar, D., Horváth, C., Keresztes, L., Jansen, S., Becker, N., Kaiser, A., Popescu, O., Schmidt-Chanasit, J., Jöst, H. and Lühken, R. (2016) Pilot longitudinal mosquito surveillance study in the Danube Delta Biosphere Reserve and the first reports of *Anopheles algeriensis* Theobald, 1903 and *Aedes hungaricus* Mihályi, 1955 for Romania. *Parasites & Vectors* 9(1): 196. – Impact Factor 2016: 3.035 (AIS 2016: 0.878; Q1)

Tomazatos, A., Cadar, D., Török, E., Horvath, C., Maranda, J., Keresztes, L., Spinu, M., Jansen, S., Jöst, H., Schmidt-Chanasit, J., Tannich, E. and Lühken, R. Circulation of *Dirofilaria immitis* and *Dirofilaria repens* in the Danube Delta Biosphere Reserve, Romania *Parasites & Vectors* 11(1): 392. – Impact Factor 2017: 3.163 (AIS 2017: 0.972; Q1)

Accepted papers

Török, E., Kolcsár, L.P., Keresztes, L. Jansen, S., Girbea, C., Popescu, O., Schmidt-Chanasit, J. and Keresztes L. (2018) Faunistic surveys on Culicidae (Diptera) and their arboviruses in the area of a metropolis, Cluj-Napoca, Romania, *North-Western Journal of Zoology*. – Impact Factor: 0.733

Török E., Ujvárosi B.L., Kolcsár L.P. and Keresztes L. (2018) DNA Barcode Library of the Romanian Culicidae (Insecta, Diptera) with a revised checklist and new faunistic data, *Studia Universitatis Babeş – Bolyai, Biologica* – ISSN: 1221-8103

The following article are waiting to be published focusing on my PhD thesis issues:

Török, E., Kolcsár, L.P. and Keresztes, L.: New records and faunistic data of mosquitoes (Diptera, Culicidae) from Albania, Hungary, Macedonia, Montenegro and Serbia, *Turkish Journal of Zoology*, ZOO-1803-23 – Impact Factor: 0.785

Our results were presented at the 8th International Dipterological Conference of Central Europe held between September 28 to 30, 2015 in Kežmarské Žľaby in Tatras, Slovakia. At this conference I presented a poster having the title: „*Longitudinal study mosquito surveillance in the Danube Delta and the first report of Ochlerotatus hungaricus (Mihalyi, 1955) for Romania*". The abstract was published in the conference proceedings (in Hamerlík Ladislav, Dobríková Daniela & Stoklasa Jaroslav 2015. The 8th Central European Dipterological Conference, Conference Abstracts, (Kežmarské Žľaby, 28th – 30th September, 2015, Banská Bystrica: Belianum, ISBN 978-80-557-0919-2, pp. 74.). Some preliminary results were also presented at the International Conference on Zoology and Zoonoses, held between 26 to 28 October, 2016 in Hissar, Bulgaria. At this conference I presented a poster with the title: „*Mosquito (Culicidae, Diptera) and vector monitoring in Danube Delta most representative ecosystems 2014-2015 (Romania)*", as well as at the „17 Biology Days", at 8-9 April, 2016, Cluj-Napoca. My presentation title was: "*Preliminary results of the mosquito (Culicidae, Diptera) survey in Danube Delta, using integrative taxonomy tools*".

Additionally, our results were also presented at the International Conference in Debrecen, Hungary: Interdisciplinary Carpathian Basin ELTE MASZ PhD-Conference, held 6 April, 2018. At this conference I presented my PhD thesis most relevant results, having the title: „*Longitudinal study mosquito (Diptera, Culicidae) surveillance in the Danube Delta Biosphere Reserve and identification of their exotic viruses*".

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