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Faunistic survey on Culicidae (Diptera) and their arboviruses in the area of a metropolis Cluj-Napoca, Romania

Edina TÖRÖK[1,3,4,5,*], Levente-Péter KOLCSÁR[1], Octavian POPESCU[2,3,4] and Lujza KERESZTES[1]

1. Hungarian Department of Biology and Ecology, Center of Systems Biology, Biodiversity and Bioresources, Babeş-Bolyai University, 400006 Cluj-Napoca, Clinicilor 5-7, Romania.
2. Faculties of Biology and Geology, Babeş-Bolyai University, 400006 Cluj-Napoca, Clinicilor 5-7, Romania.
3. Romanian Academy Institute of Biology, Splaiul Independenţei 296, 060031 Bucureşti, Romania.
4. Interdisciplinary Research Institute on Bio–Nano–Sciences of Babeş–Bolyai University, Treboniu Laurian 42, 400271, Cluj-Napoca, Romania.
5. MTA Centre for Ecological Research, Institute of Ecology and Botany, Lendület Landscape and Conservation Ecology, Alkotmány u. 2-4, H-2163 Vácrátót, Hungary.
* Corresponding author, E. Török, E-mail: edinatorok7@gmail.com

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Abstract. A number of human and animal diseases can be caused by viruses, which are transmitted by arthropods (e.g. arthropod-borne virus). These arthropod vectors show a growing importance in many European countries, including Romania. Globalization and climate change creates favorable conditions for several exotic pathogens to enlarge their original ranges toward temperate regions. Our research aim is to reveal mosquito species which could be potential vectors of mosquito-borne viruses (e.g. mobovirus). In this study, we are focusing on different wet ecosystems in a metropolis with important human concentration, such as Cluj-Napoca. Using a standard collection method (gravid traps), we performed a longitudinal survey to identify and describe mosquito communities as well as to detect moboviruses occurring in the area. During our work, molecular tools were used, such as pan-PCR and cell cultures. A total number of 728 Culicidae individuals were collected, belonging to 14 different species. We found 11 vector species, which are potential transmitters of mobovirus. Nine species are reported for the first time around Cluj-Napoca, based on our results. *Coquiuettidia richiardii* is reported here for the first time from Transylvania. The high number of Culicidae species identified in the area of Cluj Napoca is likely to be the result of the presence of a large variety of suitable habitats. Despite the high number of specimens collected, all samples were negative to arboviruses, thus the Culicidae fauna of the area show a low significance in medical importance.

Key words: faunistic data, urban mosquitoes, new records, vector species, mosquito-borne viruses.

Introduction

Estimation of arthropod diversity around human settlements has been becoming one of the major issues of medical entomology as numerous human and animal diseases caused by viruses are transmitted by such animals and their early detection are of growing importance in many European countries, including Romania. For example mosquito (Culicidae) species are also able to inoculate a series of other pathogens (namely, mosquito-borne viruses the shorthand moboviruses) during blood feeding on an infected donor and to deliver to a recipient host, including humans (Hubálek 2008). In Europe, a series of recent works identified repetitive presence of some moboviruses responsible for severe diseases in animals and humans which were once characteristics of the tropical areas, such as West Nile virus, Sindbis virus, Usutu virus, Ťahyňa virus and Batai virus (Hubálek 2008, Jöst el al. 2010, Sebesta et al. 2010, Jöst el al. 2011, Allering el al. 2012, Becker et al. 2012, Roiz et al. 2012). In Romania, the presence of some moboviruses, for instance West Nile Virus (Tsai et al. 1998, Reiter & Ceianu 2015) as well as Sindbis Virus (Prioteasa 2011), have been also detected. A number of human infections with West Nile Virus (Tsai et al. 1998, Reiter & Ceianu 2015) have been detected from this area, mostly around big cities with important human concentrations, like Bucureşti (Nicolescu 1998), Târgu-Mureş and Cluj-Napoca (Ungureanu et al. 1999). A human infection with West Nile Virus (WNV) was reported in Romania in 1950 for the first time, and additionally in 1955 in central Transylvania. Adriana Neghina and Raul Neghina detected two human cases in Cluj-Napoca by 2011. Similarly, the ECDC Annual epidemiological report (http://ecdc.europa.eu/en/healthtopics/west_nile_fever/West-Nile-fever-maps/pages/index.aspx) also reported an additionally new West Nile human infection case from Romania in 2014 and 2016. West Nile Virus infected wild birds observed around Cluj-Napoca (Paştiu et al. 2016). However, WNV infected mosquitoes are reported only from Bucureşti (Dinu et al. 2015). These results suggest that *Culex pipiens* and *Cx. modestus* are the most important WNV vectors in Romania (Nicolescu 1998) as well as in other parts of Europe, along with a number of other species such as *Anopheles maculipennis* s.l., *An. claviger, Aedes cinereus*, *Ae. vexans*, *Coquillettidia richiardii*, *Cx. territans, Culiseta alaskaensis, Cu. annulata*, *Ochlerotatus annulipes*, *Oc. cantans*, *Oc. caspius*, *Oc. Cataphylla* and *Oc. punctor* (Savage et al. 1999, Balenghien et al. 2008, Sambri et al. 2013, Dinu et al 2015). In Romania, there are a number of 58 Culicidae species described so far, based mostly on morphological characters (Nicolescu 1994, Nicolescu et al. 2002, Nicolescu et al. 2003a, Nicolescu et al. 2003b, Nicolescu et al. 2003c, Nicolescu et al. 2007, Török et al. 2016). Previous data have shown the importance of these species as vectors of moboviruses in our country, and a number of 18 species were already detected as vectors for important pathogens (Purcarea-Ciulacu 2008, Prioteasa 2011).

Longitudinal monitoring studies on mosquito populations will help us understand certain aspects of the ecology of moboviruses and their genetic and phylogenetic characterization in this biogeographically important area, like the Carpathian region. Cluj-Napoca is located in the hilly region of the Carpathians along the drainage basin of Someşul Mic River. In this area, many important natural or semi natural and artificial wet ecosystems occur, which were never monitored in the case of Culicidae. The importance of such inves-

tigations is highlighted also by some previous WNV occurrence data such as human and bird pathogen from the area (Neghina & Neghina 2011, Paştiu et al. 2016).

The present research aims to identify mosquito species and their vector borne viruses in different natural and artificial ecosystems in Cluj-Napoca and its surrounding in respect to their role as vectors of some important moboviruses.

Material and methods

In our present study, we applied a survey to detect Culicidae communities from different ecosystems in Cluj-Napoca, in 2015. Mosquitoes were sampled from May to September, from 7 study sites from different wet or humid ecosystems, as follows (Fig. 1.):

1. The Someşul Mic riverside near "Eastern Hills of Cluj" hayfields, GPS coordinates 46°47'22.74"N, 23°36'1.47"E (Fig. 2, 1.)
2. Riparian vegetation along a secondary channel of Someşul Mic river near "Eastern Hills of Cluj "hayfields, GPS coordinates(46°48'2.80"N, 23°36'24.38"E) (Fig. 2, 2.)
3. "Lacul 3" urban lake, GPS coordinates (46°46'28.85"N, 23°37'48.58"E) (Fig. 2, 3.)
4. Fishing lake, GPS coordinates (46°46'40.34"N, 23°38'44.34"E) (Fig. 2, 4.)
5. Water Treatment Station in center of Cluj Napoca, GPS coordinates 46°45'53.05"N, 23°33'4.56"E (Fig. 2, 5.)
6. Botanical Garden, GPS coordinates 46°45'41.49"N, 23°35'10.93"E (Fig. 2, 6.)
7. Forest habitat in "Făgetul Clujului – Valea Morii, GPS coordinates 46°45'25.44"N , 23°33'52.94"E (Fig. 2, 7.)

Handmade Gravid Traps (GT) were used, 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 (Fig. 2.). The GT were used for 17 weeks and the mosquitoes were collected weekly and preserved at -20 C° for later identification.

Culicidae material was identified at the Hungarian Department of Biology and Geology, (Babeş-Bolyai University, Cluj-Napoca) based on morphological characteristics using identification keys (Becker et al. 2010, Kenyeres & Tóth, 2008) as well as an identification program software (Schaffner et al. 2001). Specimens were examined with an Olympus SZ50 microscope, and during specimen identification ice was used as substrate. The identification of the potential viruses was performed in the laboratories of the Arbovirology Group, Bernhard Nocht Institute for Tropical Medicine (BNITM) in Hamburg.

Mosquitoes were placed in sterile 2 ml reaction tubes and 1.5 ml of cell culture medium (high-glucose Dulbecco's modified Eagle's medium [Sigma-Aldrich, St. Louis, MO] with 10 % heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B) and 0.75 µl Ziconia beads (Biospec; 2.0 mm beads) were added for homogenization in a TissueLyser (Qiagen, Hilden, Germany) for 2 min minutes at 50 oscillation/s. The suspensions were clarified by centrifugation (5,000 g for 1 min), and the supernatant was used for DNA extraction with the RTP Pathogen Kit (Stratec Biomedical AG, Birkenfeld, Germany) according to the manufacturer's instructions. After extraction, PCR was used and reaction was performed with the HotStartTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol (Török et al. 2016). The classical pan-PCR method was applied to detect members of families or genera of moboviruses (Flaviviridae, Orthobunyaviridae, Alphavirus, Rhabdoviridae and Phebovirus).

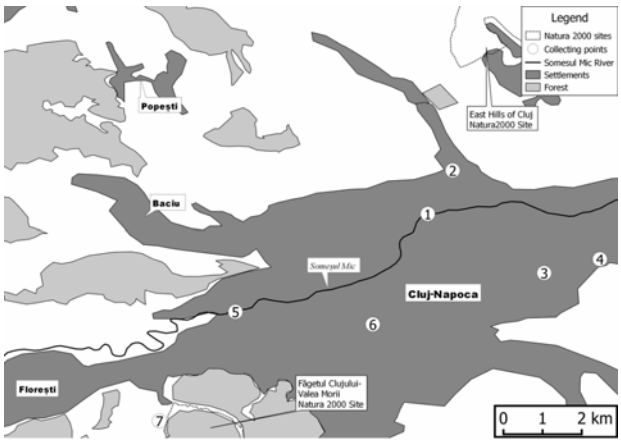

Figure 1. Collecting sites in Cluj-Napoca.

Figure 2. Collecting habitats and Gravid traps in Cluj-Napoca.

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, Sivaram et al. 2010) with an impaired RNAi pathway. After infection we incubated mosquito cell cultures, and after the multiplication of viral genome we amplified and sequenced using adequate protocols. After the infarction we added 200 ml Schneider's Drosophila Medium in every third day on the cell cultures and incubated two weeks in 28 °C temperatures (Igarashi 1978). After 2 weeks, cells were analyzed with the microscope and check in the cytopathic effects (CPE). In case we found CPE we repeated the extraction and panPCR analyses.

Viral sequences were compared with existing viral genome, extracted from the GenBank database checking for mutations in the nucleotide and amino acid sequences.

Results and Discussion

During the present study, we collected 728 specimens belonging to 14 different mosquito species (Table 1). The majority of the mosquito materials were collected at six differ-

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

ent collection sites. The highest amount was collected in the Botanical Garden (number 6) (165 specimens) and in forest habitats (no. 7) (155 specimens). In the perimeter of the Water Treatment Station site we were not able to detect any Culicidae species, probably due to the periodical control of mosquitoes in the area. 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) and resulting in a number of 14 Culicidae species, which represents 46.66% of species identified from Transylvania. This is a relatively high number compared to recent report of Nicolescu et al. (2002, 2003), who identified 30 Culicidae species in Transylvania. *Coquiuettidia richiardii* is here reported for the first time from Transylvania. Another eight species are collected first time in and around Cluj-Napoca: *An. claviger, Cu. alaskaensis, Cu. annulata, Ae. cinereus, Oc. annulipes, Oc. cantans, Oc. cataphylla* and *Oc. punctor*.

Coquiuettidia richiardii is an abundant species in lakes but also occurring in surroundings of fresh waters, old river beds and estuaries. Larvae have long development time (up to 10 months) and require the presence of bulrush species (Tóth 2004). Females, which are abundant and severe nuisance to humans and domestic animals, have frequently been reported (Becker et al. 2010).

An. claviger was found in cool water in shaded areas, as they are known to require fresh and clean water. The larvae are often found along with *Oc. punctor*. The larvae occur among the aquatic vegetation on the margins of small well in mountain streams. The imagoes do not appear 100 m away from breeding sites. These mosquitoes are important malaria vectors in Near East and Central Asia (Becker et al. 2010).

Culiseta alaskaensis is a typical forest species, adapted to cold, rich after snow-melt. The females leave their winter habitats usually earlier than other vertebrate feeding mosquito species. *Culiseta alaskaensis* is a well-researched species in the tundra zone (Becker et al. 2010).

Culiseta annulata hibernate in cellars, attics of dwellings or in sheds of domestic animals. In these habitats they can be extremely numerous during wintertime, when the hibernation is interrupted by rising temperatures or humidity. Winter habitats can also be found far away from human settlements in tree cavities, stacks of wood, or in other natural shelters (Becker et al. 2010).

Aedes cinereus larvae prefer forest habitats, while the imagoes tend to occur in different habitats, such as breeding sites with long migration range. These species are occurring in masses (Tóth 2004).

Ochlerotatus annulipes like sunshine, breed in open meadow pools at forest edges and inside deciduous forests, preferably in semi-permanent pools with leaf detritus, where they are often found together with larvae of *Oc. cantans* (Tóth 2004).

Oc. cantans is a forest species and has a long life. Flying period is from early spring until the end of summer. The daily biting cycle of this species on humans seems to be bimodal with peaks during dawn and dusk (Tóth 2004).

Oc. cataphylla is present in forest ecosystems, living in hills and mountain regions. *Oc. cataphylla* is an all-day active species (Tóth 2004).

Oc. punctor is a snow-melt mosquito, which has a preference for swampy forests with boggy waters. This species is a Holarctic species complex, which is present in Europe with three species, *Oc. hexodontus*, *Oc. punctodes* and *Oc. punctor*. *Oc. punctor* can be qualified as a cold stenotherm, all-day active species (Becker et al. 2010).

The high number of Culicidae species identified in the area of Cluj-Napoca is probably due to the presence of the high variety of suitable habitats, mostly wet habitats with important organic supply. Biological knowledge of the 14 collected mosquito species is summarized in Table 2.

Among the different Culicidae species identified in this study, 11 species (*Aedes vexans*, *Anopheles claviger*, *An. maculipennis* complex, *Culex pipiens*, *Cx. territans*, *Culiseta annulata*, *Ochlerotatus annulipes*, *Oc. cantans*, *Oc. caspius*, *Oc. punctor*, *Coquiuettidia richiardii*) are known to be potential vectors for a series of mosquito-borne viruses [(e,g, Sindbis Virus (*Togaviridae*), West Nile Virus, Usutu Virus (*Flaviviridae*), Tahyna Virus (*Banyaviridae*)] (Hubálek 2008, Rossati et al. 2015). During the present study, we were not able to detect any of the viruses mentioned above, having no positive results applying pan-PCR. Despite the fact that we were not able to identify viruses in the collected mosquito material, we inoculated the homogenized samples on C6/36 *Ae. albopictus* cell cul-

Table 2. Mosquito taxa recorded in the study area of Cluj-Napoca in Romania during the sampling period in 2015 with the number of specimens collected, their respective overall proportion, and biological information.

Taxa	Oviposition sites	Overwintering stage	No. of generations	Feeding preference	Involved in mobovirus transmission	Reference
Anopheles claviger (Meigen, 1804)	water	larvae	multivoltine	human and other mammals	Batai virus, Tahyna virus	Schäfer et al. 2004, Hubálek 2008, Kenyeres and Tóth 2008, Becker et al. 2010
Anopheles (Anopheles) maculipennis complex Meigen, 1818	water	females	multivoltine	human and other mammals	Batai virus, Tahyna virus, West-Nile virus	Schäfer et al. 2004, Hubálek 2008, Kenyeres and Tóth 2008, Becker et al. 2010
Aedes (*Aedes*) *cinereus* Meigen 1818	land	eggs	multivoltine	human and other mammals	Sindbis virus, Tahyna virus	Schäfer et al. 2004, Hubálek 2008, Kenyeres and Tóth 2008, Becker et al. 2010
Aedes (*Aedes*) *vexans* (Meigen, 1830)	land	eggs	multivoltine	human and other mammals	Tahyna virus, West-Nile virus, Lednice virus	Hubálek 2008, Kenyeres and Tóth 2008, Becker et al. 2010, Bueno-Marí and Jiménez-Peydró 2011
Culex (Culex) pipiens Linnaeus, 1758	water	females	multivoltine	human and aves	Batai virus, Tahyna virus, West-Nile virus, Sindbis virus	Hubálek 2008, Kenyeres and Tóth 2008, Becker et al. 2010
Culex (Culex) territans Walker, 1856	water	females	multivoltine	amphibian and aves	Sindbis virus	Hubálek 2008, Kenyeres and Tóth 2008, Becker et al. 2010, Bueno-Marí and Jiménez-Peydró 2011
Culiseta (Culiseta) alaskaensis (Ludlow, 1906)	water	females	multivoltine	human and other mammals	-	Kenyeres and Tóth 2008, Becker et al. 2010
Culiseta (Culiseta) annulata (Schrank, 1776)	water	females	multivoltine	human, aves and other mammals	-	Schäfer et al. 2004, Kenyeres and Tóth 2008, Becker et al. 2010
Aedes (Ochlerotatus) annulipes (Meigen 1830)	land	eggs	univoltine	human and other mammals	Tahyna virus	Hubálek 2008, Kenyeres and Tóth 2008, Becker et al. 2010
Aedes (Ochlerotatus) cantans (Meigen, 1818)	land	eggs	univoltine	human and other mammals	Tahyna virus, West-Nile virus	Schäfer et al. 2004, Hubálek 2008, Kenyeres and Tóth 2008, Becker et al. 2010
Aedes (Ochlerotatus) caspius (Pallas, 1771)	land	eggs	multivoltine	human and other mammals	Tahyna virus, West-Nile virus	Hubálek 2008, Kenyeres and Tóth 2008, Schäfer et al. 2004, Becker et al. 2010
Aedes (Ochlerotatus) cataphylla (Dyar, 1916)	land	eggs	univoltine	human	-	Schäfer et al. 2004, Hubálek 2008, Kenyeres and Tóth 2008, Becker et al. 2010
Aedes (Ochlerotatus) punctor (Kirby 1837)	land	eggs	univoltine	human, aves and other mammals	Tahyna virus, West-Nile virus, Inkoo virus	Schäfer et al. 2004, Hubálek 2008, Kenyeres and Tóth 2008, Becker et al. 2010
Coquiuettidia (Coquillettidia) richiardii (Ficalbi, 1889)	water	larvae	univoltine	human and other mammals	Batai virus, Tahyna virus, West-Nile virus	Hubálek 2008, Kenyeres and Tóth 2008, Becker et al. 2010, Bueno-Marí and Jiménez-Peydró 2011

tures. After two weeks of incubation time, we analyzed and found evidences of cytopathogenic effects (CPE) in 16 samples from which we extracted and analyzed genetic material again with pan-PCR. The results showed the presence of non-specific mosquito viruses.

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 from here. There are a high number of important suitable wet habitats in Cluj Napoca and its surroundings (lake ecosystems, the presence of the Somes 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.

However, a high number of Culicidae species (11 out of 14) identified by us in the Cluj-Napoca area represent 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 estimate their frequency in mosquitoes and implications in human wellbeing.

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