

## UPDATE FOR LITHUANIAN *CULICOIDES* (DIPTERA: CERATOPOGONIDAE) FAUNA LIST WITH RECORDS OF FIVE NEW SPECIES

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### Introduction

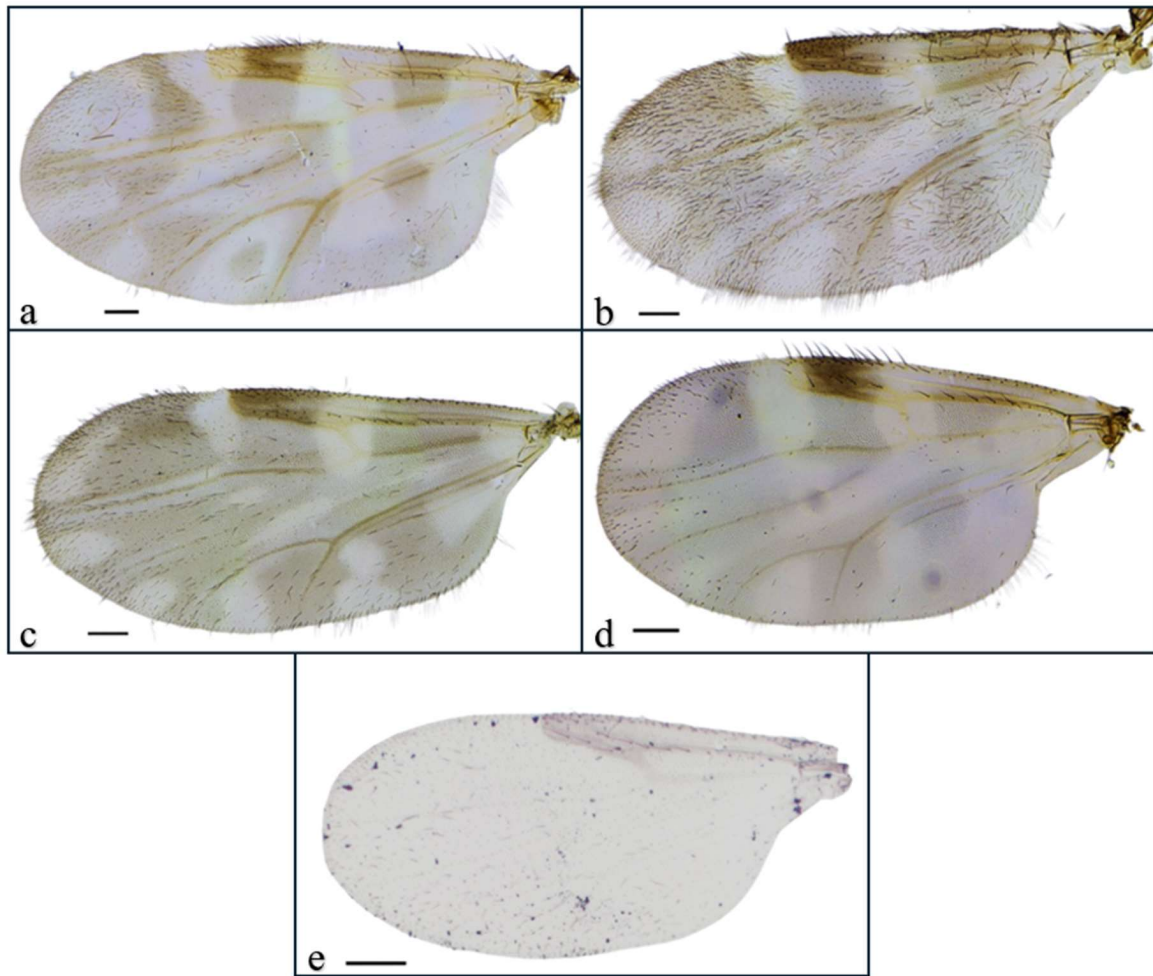
*Culicoides* biting midges are known vectors of several viruses, bacteria, nematodes, and protozoa worldwide; because of their blood-feeding habit, they are of great importance in the field of medical and veterinary entomology (Carpenter *et al.*, 2013). Although it is currently known that globally, the genus *Culicoides* includes 1347 species (Borkent & Dominiak, 2020), the majority of biting midge fauna remains under-researched. In Lithuania, 27 species of *Culicoides* were earlier known (Pakalniškis *et al.*, 2006) and two additional records were added in 2023 (Bernotienė *et al.*, 2023). Here, we report five species of *Culicoides* that are new to the Lithuanian fauna.

### Material and Methods

Biting midges were collected during several studies around Lithuania and in different localities: near Juodkrantė village on the Curonian Spit located in the Baltic Sea; in a horse stables “Horseland” in Kunkiai, Klaipėda district; the Lithuanian Zoological Garden in Kaunas; Mažeikiai near the river Venta and a small pond. Specimens in these localities were collected using Biogents UV light traps, which were installed at 1.5–2 meters above the ground around 2–3 hours before sunset and were removed the next day 2–3 hours after sunrise. Material was collected into plastic containers with water and a drop of liquid soap and, on the same day, transported to the laboratory for further investigation.

Biting midges were also collected using an entomological net put through an open window of a car driven on the road (Malmqvist *et al.*, 2004) along the Vilnia river in Belmontas district of Vilnius. Collected specimens were transported to the laboratory the same day for further identification.

Biting midges were identified using an interactive identification key for *Culicoides* (Diptera: Ceratopogonidae) females from the Western Palaearctic region (Mathieu *et al.*, 2012) and dissected for parasite investigation. The cytochrome oxidase subunit I gene sequences (*COI*) of several specimens were barcoded by PCR based methods using primers LCO1490/HCO2198 or C1-J-1718/C1-N-2191 and analyzed using Geneious Prime® 2024.0.7 software (<https://www.geneious.com>) and BOLD system (<https://v3.boldsystems.org/>). Specimens are preserved at Nature Research Centre (Vilnius, Lithuania) and at the Institute of Bacteriology and Parasitology, Medical Faculty, University of Strasbourg (Strasbourg, France).



**Fig 1.** Wings of (a) *C. lupicaris*, (b) *C. duddingstoni*, (c) *C. clastrieri*, (d) *C. dewulfi*, (e) *C. minutissimus*. Scale - 100  $\mu\text{m}$  (original, author M. Kazak)



**Fig. 2.** *C. festivipennis* (left) and *C. clastrieri* (right) thorax color difference (*C. clastrieri* thorax has a lighter pattern): *C. clastrieri* thorax pattern is lighter than *C. festivipennis*, which can be seen without dissection (original, author M. Kazak)



**Fig. 3.** *C. dewulfi* short sensilla near the sclerotised plaque on the first segment of the abdomen (original, author M. Kazak)

**List of localities**

Locality	Administrative district	Coordinates (LAT, LONG)
Augustaičiai, Ventos RP <sup>a</sup>	Mažeikiai distr.	56.14023, 22.79965
Belmontas <sup>b</sup>	Vilnius mun.	54.68128, 25.31499 - 54.68361, 25.35530
Burbiškės <sup>a</sup>	Mažeikiai distr.	56.14361, 22.82172
Juodkrantė <sup>a</sup>	Neringa mun.	55.55676, 21.12398
Kairėnai, Vilnius University Botanical Garden	Vilnius mun.	54.73304, 25.40083
Kaunas, Lithuanian Zoological Garden <sup>a</sup>	Kaunas distr.	54.90402, 23.95511
Kunkiai <sup>a</sup>	Klaipėda distr.	54.90402, 23.95511
Puvočiai	Varėna distr.	54.11582, 24.30294

<sup>a</sup> light trapping, <sup>b</sup> car-net collection

**List of Species*****Culicoides (Avaritia) dewulfi* Goetghebuer, 1936 (Fig 1. d)**

Kunkiai, night from 11<sup>th</sup> till 12<sup>th</sup> of July, 2024, 9♀.

***Culicoides (Culicoides) lupicaris* Downes & Kettle, 1952 (Fig 1. a)**

Juodkrantė, night from 16<sup>th</sup> till 17<sup>th</sup> of August, 2022, 2♀; night from 17<sup>th</sup> till 18<sup>th</sup> of August, 2022, 6♀; Kunkiai, night from 11<sup>th</sup> till 12<sup>th</sup> of July, 2024, 6♀.

***Culicoides (Oecacta) duddingstoni* Kettle & Lawson, 1955 (Fig 1. b)**

Juodkrantė, night from 21<sup>st</sup> till 22<sup>nd</sup> of July, 2022, 2♀; night from 16<sup>th</sup> till 17<sup>th</sup> of August, 2022, 6♀ (GenBank accession no PQ524999, PQ525000).

***Culicoides (Sensiculicoides) clastrieri* Callot, Kremer & Dedit, 1962 (Fig 1. c)**

Kaunas, night from 28<sup>th</sup> till 29<sup>th</sup> of June, 2023, 5♀, night from 1<sup>st</sup> till 2<sup>nd</sup> of August, 2023, 1♀; Kairėnai, night from 4<sup>th</sup> till 5<sup>th</sup> of July, 2023, 1♀; Mažeikiai, night from 23<sup>rd</sup> till 24<sup>th</sup> of June, 2024, 67♀; night from 17<sup>th</sup> till 18<sup>th</sup> of July, 2024, 79♀; Puvočiai, night from 12<sup>th</sup> till 13<sup>th</sup> of June, 2023, 1♀, night from 14<sup>th</sup> till 15<sup>th</sup> of June, 2023, 1♀.

***Culicoides (Wirthomyia) minutissimus* (Zetterstedt, 1855) (Fig 1. e)**

Belmont, the road alongside Vilnia river, the day of 17<sup>th</sup> of July, 2023, 1♀ (GenBank accession no PQ606571).

**Discussion**

Specimens from two out of five newly found species (*Culicoides minutissimus* and *C. duddingstoni*) were identified using both morphological and molecular methods. The other three species, namely, *C. lupicaris*, *C. dewulfi* and *C. clastrieri*, have been identified based on the observation of the full morphological characters under the microscope. Unfortunately, the molecular attempts for sequencing specimens of these species were not successful. In any case, while it would be informative for *C. lupicaris* and *C. dewulfi*, *C. clastrieri* cannot be distinguished from *C. festivipennis* using molecular methods as studies have shown a nearly identical COI sequences (Hadj-Henni *et al.*, 2021; Sarvašová *et al.*, 2014). The first records of *C. duddingstoni* and *C. lupicaris* were made in Juodkrantė,

from material collected in 2022. From eight morphologically identified *C. duddingstoni* specimens, six were confirmed using PCR-based methods and sequences presented 100% similarity with *C. duddingstoni* deposited sequences (GenBank accession No. OL702741).

There is a lack of information regarding biology of *C. duddingstoni*; a few studies have found some larvae of this *Culicoides* species in a muddy unvegetated area near a pond and in standing water with *Typha* spp. roots (González *et al.*, 2013). This species is characterized by ornithophilic feeding behavior and bloodmeal research have shown that *C. duddingstoni* prefers to feed on passerine birds (Augot *et al.*, 2017; González *et al.*, 2013; Martínez-de La Puente *et al.*, 2015).

Some species of biting midges have characteristic wing patterns and/or other specific features (differences in head and leg structures, spermatheca size and number), which allow them to be easily distinguished from other species only by morphology (Mathieu *et al.*, 2012). This is the case with three newly found species in our investigation: *C. lupicaris*, *C. clastrieri* and *C. dewulfi*.

Specimens were attributed to *Culicoides lupicaris* based on the wing pattern and observation of other characters (Mathieu *et al.*, 2012; Ramilo *et al.*, 2013). *C. lupicaris*, together with another species *C. pulicaris*, are included into the Pulicaris complex. These species develop in small water-logged substrates near lakes, bogs, small marshy places, forest leaf-litter, and ponds (Kremer, 1965; Kettle & Lawson, 1952; Konurbayev, 1965; Trukhan, 1975; Mirzaeva *et al.*, 1976; Tweddle, 2002). González *et al.* study (2013) showed that *C. lupicaris* can be more abundant than *C. pulicaris* in terrestrial farm microhabitats. Both species have mainly mammalophilic feeding behaviour (Augot *et al.*, 2017; Martínez-de La Puente *et al.*, 2015). It was expected that *C. lupicaris* would be found near horse stables where the hosts for possible bloodmeal would be easily accessible. Additionally, there were artificial ponds in the “Horseland” area close to the horse stables, which is an ideal breeding site for *C. lupicaris*.

*Culicoides clastrieri* is very similar to *C. festvipennis*, but they can be distinguished by the presence of *sensillae coeloconia* (present on antennal segments 3, 11–15 for *C. clastrieri* and 3–15 for *C. festvipennis* (Mathieu *et al.*, 2012)) on antenna segments and by the color and pattern differences present in their thoraxes (Fig. 2) (Sarvašová *et al.*, 2014) and by the elongated pale spot in the m cell of the wing (Mathieu *et al.*, 2012). However, some differences were found in biology of these two species, noting that *C. clastrieri* prefers habitats for breeding with higher organic matter when compared with *C. festvipennis* (Kremer *et al.*, 1978). We found six specimens of this species in the Lithuanian Zoo in an enclosure where ducks (Anatidae) are kept and near the enclosure with wolves, in an open area and close of a small river. The enclosure with ducks has an artificial pond with a lot of organic matter on its banks, which can be a good breeding site for this species. The other places where *C. clastrieri* specimens were caught had small streams near the trapping location, and forested area provided a lot of organic matter on the ground, which could create suitable breeding spots. Augot *et al.* (2017) have shown that *C. clastrieri* is ornithophilic/mammalophilic and might feed on both birds from families Anatidae and Turdidae and on humans.

Within the *Avaritia* species, *Culicoides dewulfi* has unique combination of features on wings and spermatheca, which can be used to distinguish it from other (Mathieu *et al.*, 2012). Moreover, *C. dewulfi* identification can be supported by the high number of hairs (8-12) near the sclerotized plaque on the first segment of the abdomen (Fig. 3) (Campbell & Pelham-Clinton, 1960). *Culicoides dewulfi* is known for mammalophilic behaviour and

several studies found it to be feeding mainly on farm animals, such as cows (*Bos taurus*), sheep (*Ovis aries*), wild boars (*Sus scrofa*), European rabbits (*Oryctolagus cuniculus*), and horses (*Equus caballus*) (Martínez-de La Puente *et al.*, 2015; Santiago-Alarcon *et al.*, 2013). Larvae of *C. dewulfi* can be found in decomposing cattle dung and in dung of mixed composition (Werner *et al.*, 2020). We found at least nine specimens in the material collected close to the horse stables, which is a suitable environment for larva breeding and adult feeding. However, since only a few studies have been carried out in Lithuania on locations with domestic animals, it might be that *C. dewulfi* is more widespread in Lithuania than was previously thought. More research should be carried out on this issue throughout the country.

Despite of the trapping effort, only one individual of *C. minutissimus* was collected. Although *C. minutissimus* is widespread in Europe, it is usually present in low numbers and is rarely caught; although recent study found that midges of this species prefer to inhabit mixed grasslands or wetlands without bushes and trees as well as the banks of rivers and lakes (Werner *et al.*, 2020). A study of Votýpka *et al.* (2009) suggested that *C. minutissimus* is an ornithophilic species and might repeatedly enter bird nest boxes to feed on nestlings of passerine birds. We were able to identify this specimen morphologically and we obtained *COI* sequence although there were no other sequences deposited in GenBank for this species before. This is the first *COI* sequence of *C. minutissimus* deposited in GenBank.

Generally *Culicoides* insects are characterized by adaptation to use a wide spectrum of different habitats (aquatic and semiaquatic) for breeding which range from the tropics to the arctic tundra and use a broad variety of habitats rich in organic matter (Mullen & Durden, 2019). Specific ecological and biological features can differ highly between different species; therefore to cover the full range of species diversity in studied areas it is necessary to plan the trapping in a way that covers as wide a range of habitats as possible. Over the last few years, research on biting midges in Lithuania have been expanded to new, previously unexplored areas, nearby farms or in a zoo, which could explain the discovery of seven new species of *Culicoides* for Lithuania over the last few years. With this study, it was possible to expand the list of *Culicoides* in Lithuania (Pakalniškis *et al.*, 2006; Bernotienė *et al.*, 2023) to 34 species. Despite this, many other areas and regions of Lithuania remains poorly studied for biting midges and research paired with monitoring of these insects should be encouraged.

The knowledge on the *Culicoides* species composition is important for understanding the areas of occurrence of these insects, and potential hot spots for pathogen transmission. This would also allow to better understand the distribution through the years in a backdrop of global climate change.

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**Lietuvos *Culicoides* (Diptera, Ceratopogonidae) rūšių sąrašo papildymas 5 naujomis rūšimis**

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**Santrauka**

Straipsnyje pateikiami duomenys apie penkias naujas genties *Culicoides* (Diptera: Ceratopogonidae) rūšis Lietuvos faunai, aptiktas keliose skirtingose Lietuvos vietovėse atliekant sezoninius entomologinius tyrimus: *Culicoides dewulfi*, *C. lupicaris*, *C. duddingstoni*, *C. clastrieri* ir *C. minutissimus*. Visos rūšys buvo identifikuotos pagal morfologinius požymius, dviejų rūšių identifikavimas patvirtintas molekuliniais metodais (DNR COI sekų analize), likusių trijų rūšių apibūdinimui užteko morfologinio apibūdinimo ir/arba molekulinis apibūdinimas buvo neįmanomas. Aptiktos rūšys pasižymi skirtingomis ekologinėmis savybėmis: *C. lupicaris*, *C. clastrieri* ir *C. minutissimus* vystosi šalia vandens telkinių (*C. lupicaris* nedideliuose vandeninguose substratuose prie ežerų, pelkių, miško lapų paklotėje ir tvenkiniuose; *C. clastrieri* upės pakrantėse su didesniu kiekiu organinių medžiagų, kurių pakraščiai yra daugiau ar mažiau apžėlę įvairiais augalais; *C. minutissimus* veisiasi mišrioje pievoje arba pelkėse be krūmų ir medžių, taip pat upių ir ežerų pakrantėse, o *C. duddingstoni* veisiasi dumblėtose neapaugusiose teritorijose prie tvenkinių ar stovinčio vandens su *Typha* spp. augmenija). *Culicoides dewulfi* pageidaujamas veisimosi substratas yra pūvantis galvijų mėšlas ir mišrios sudėties mėšlas. Šios rūšys taip pat skiriasi savo mitybiniais ypatumais – *C. lupicaris* ir *C. dewulfi* yra mamalofilinės rūšys, kurios renkasi žmones ir naminius gyvulius kaip maisto šaltinį, tuo tarpu *C. duddingstoni* ir *C. minutissimus* yra ornitofilinės rūšys, kurios maitinasi tik paukščių krauju, o *C. clastrieri* maitinasi ir žmonių, ir paukščių krauju.

Smulkieji mašalai pasaulyje žinomi kaip įvairių patogenų platintojai gyvūnams ir žmonėms, todėl jų įvairovės ir gausumo tyrimai yra labai svarbūs medicininiu ir veterinariniu požiūriu.

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