Blood-meal analysis of *Culicoides* (Diptera: Ceratopogonidae) reveals a broad host range and new species records for Romania

Alexandru Tomazatos\(^1\), Hanna Jöst\(^1\), Jonny Schulze\(^1\), Marina Spinu\(^2\), Jonas Schmidt-Chanasit\(^1,3\), Daniel Cadar\(^1\) and Renke Lühken\(^1,3\)^*.

**Abstract**

**Background:** *Culicoides* biting midges are potential vectors of different pathogens. However, especially for eastern Europe, there is a lack of knowledge on the host-feeding patterns of this vector group. Therefore, this study aimed to identify *Culicoides* spp. and their vertebrate hosts collected in a wetland ecosystem.

**Methods:** *Culicoides* spp. were collected weekly from May to August 2017, using Biogents traps with UV light at four sites in the Danube Delta Biosphere Reserve, Romania. Vectors and hosts were identified with a DNA barcoding approach. The mitochondrial cytochrome *c* oxidase subunit 1 was used to identify *Culicoides* spp., while vertebrate hosts were determined targeting cytochrome *b* or 16S rRNA gene fragments. A maximum likelihood phylogenetic tree was constructed to verify the biting midge identity against other conspecific Palaearctic *Culicoides* species. A set of unfed midges was used for morphological confirmation of species identification using slide-mounted wings.

**Results:** Barcoding allowed the species identification and detection of corresponding hosts for 1040 (82.3%) of the 1264 analysed specimens. Eight *Culicoides* spp. were identified with *Culicoides griseidorsum*, *Culicoides puncticollis* and *Culicoides submaritimus* as new species records for Romania. For 39 specimens no similar sequences were found in GenBank. This group of unknown *Culicoides* showed a divergence of 15.6–16.3% from the closest identified species and clustered in a monophyletic clade, i.e. a novel species or a species without reference sequences in molecular libraries. For all *Culicoides* spp., nine mammalian and 24 avian species were detected as hosts. With the exception of *C. riethi* (n = 12), at least one avian host was detected for all *Culicoides* spp., but this host group only dominated for *Culicoides kibunensis* and the unknown *Culicoides* sp.. The most common host group were mammals (n = 993, 87.6% of all identified blood sources) dominated by cattle (n = 817, 70.6%).

**Conclusions:** Most *Culicoides* spp. showed a broad host-feeding pattern making them potential bridge vectors. At the same time, new records of biting midge species for Romania, as well as a potentially unknown *Culicoides* species, highlight the lack of knowledge regarding the biting midge species and their genetic diversity in eastern Europe.

**Keywords:** *Culicoides*, Barcoding, Host-feeding patterns, Danube delta, Romania
outbreaks of the *Culicoides*-borne Oropouche virus in humans regularly occur in the Neotropics [6]. In Europe, several biting midge species are able to transmit bluetongue virus (BTV), African horse sickness virus and Schmallenberg virus (SBV) [7]. These viruses are responsible for outbreaks of non-contagious diseases in ruminants, causing huge economic losses, e.g. due to restrictions on animal trade [8].

The expansion of BTV from the Mediterranean basin to central Europe up to Scandinavia [9–11] prompted studies on *Culicoides* taxonomy [12–14], ecology [15–17] and vector competence [18–20]. In contrast, only few studies focused on the *Culicoides* fauna in southeastern Europe. Severe BTV outbreaks were observed between 2014 and 2015 in the Balkan Peninsula [21, 22]. In Romania, BTV was confirmed for the first time in 2014 [23]. The most comprehensive studies on the *Culicoides* fauna conducted in Romania date back to the end of the 20th century [24, 25]. More recent studies of the *Culicoides* fauna in Romania only focused on the known vectors of BTV. Thus, with the exception of *C. imicola* Kieffer, 1913 or *C. nubeculosus* (Meigen 1830) [26, 27], biting midges were recorded as species groups considered the most important vectors of BTV/SBV, i.e. *C. obsoletus* group and *C. pulicaris* group, or as “other *Culicoides*” [28, 29]. Currently, species-specific information on the distribution of other *Culicoides* taxa in Romania is missing.

The identification of blood sources from engorged vectors is a useful method to understand vector-host interactions and the ecology of associated pathogens [30, 31]. The host-feeding patterns of *Culicoides* have received much less attention compared to other vector groups (e.g. mosquitoes and ticks) [32, 33]. In Europe, most of the vertebrate hosts identified from engorged biting midges are ruminants [34–36]. However, other mammalian species such as humans and pigs can also be frequent [37–39]. In comparison, avian hosts are generally a more diverse, but less frequent group compared to mammals [34, 37, 38, 40]. Information about hosts of *Culicoides* species from eastern Europe was obtained by recent efforts undertaken in natural areas of Bulgaria [41] and Serbia [42]. In Serbia, blood-meal analysis predominantly detected ruminant hosts, whereas in Bulgaria, a large diversity of avian hosts was recorded for ornithophilic biting midges. To the best of our knowledge, such studies do not exist for Romania. Therefore, the aim of this study was to investigate the host-feeding patterns of *Culicoides* species collected from four sampling sites in the Danube Delta Biosphere Reserve (DDBR).

Methods

**Trapping methods and study sites**

Biting midges were collected at four sites in the DDBR as part of a pilot longitudinal arbovirus surveillance programme [43] (Fig. 1, Additional file 1: Text S1). The trapping site Letea is characterized by a semi-open enclosure for cattle and goats built of wood, reeds and rushes, located a short distance from a small canal and almost 1 km from a deciduous forest. In Sulina, the sampling site was a covered cow stable with two or three animals kept at night with a stagnant water body (canal) and a large dung heap in close proximity. The local host communities of both anthropogenic sites (Letea and Sulina) are predominantly characterized by cattle, horse, cat, poultry and humans accompanied by dogs. In contrast, the site at Dunărea Veche lays at the confluence of two branches of the Danube and adjacent small canals; a large crop field is bordered by these waters. The site Lake Roșulet is an old fishery surrounded by a shallow, stagnant canal and rows of trees isolating the area from the surrounding marshland. Only few humans (farmers and fishermen) with dogs and cats are present in Dunărea Veche and Lake Roșulet. The host community of both sites is predominantly characterized by a high diversity of wild mammals and birds.

Between May and August 2017, one Biogents Sentinel trap (BG trap; Biogents, Regensburg, Germany (http://www.biogents.com/)) equipped with an ultraviolet lamp was operated at each site for one night per week resulting in a total of 60 trap nights. The climate of the study area is continental with an annual mean temperature of 11 °C (−1 °C in January and 22 °C in July) and around 350 mm of mean precipitation per year. Sampling in the present study was conducted during a hot and dry summer. A mean temperature of 21 °C and mean precipitation under 30 mm was recorded in the Danube Delta between May and August 2017 (http://www.meteoromania.ro/clima/monitorizare-climatica/).

**Sample processing**

Insects were frozen, shipped on dry ice and stored at −80 °C in the laboratory. Due to the large amount of non-engorged and engorged *Culicoides*, only a random subsample of 1264 engorged specimens from all four sampling sites and every month of collection were selected. During the progress of sequencing, a dominance of cattle was observed for the sites Sulina and Letea. Therefore, we focused specifically on the engorged *Culicoides* from the sites Dunărea Veche and Lake Roșulet, where a wider range of wildlife host blood meals were likely to be detected. Dry, frozen storage was preferred over ethanol storage to allow virus isolation and characterization at a later time. Biting midges were separated
by engorged status and wing patterns under a stereomicroscope (Olympus ZSX12, Tokyo, Japan). In addition, a small set of unfed specimens \( (n = 37) \) from each sampling site (Sulina, \( n = 10 \); Letea, \( n = 9 \); Dunărea Veche, \( n = 10 \); Lake Roșuleț, \( n = 8 \)) were used for morphological identification, which were selected as morphologically representative for the different Culicoides species in the samples. Wings were mounted on slides in Euparal (Carl Roth, Karlsruhe, Germany) and species identified by morphology using the key of Mathieu et al. [14].

For DNA extraction, each specimen was placed into an individual sterile 2 ml tube (Eppendorf, Hamburg, Germany) with 5–9 zirconium beads (1 mm, Carl Roth) and 200 μl of Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St. Louis, MO, USA) with 100 μg/ml streptomycin (PAN-Biotech, Aidenbach, Germany) and 2.5 μg/ml amphotericin B (PAN-Biotech). The samples were homogenised with a TissueLyser II (Qiagen, Hilden, Germany) twice for 3 min at 30 Hertz. The suspension was clarified by centrifugation at 8000 \( \times \) rpm for 2 min at 4 °C. Total nucleic acid was extracted from 100 μl of supernatant, using the MagMAX™RNA/DNA Pathogen Kit with a KingFisher™ Flex Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA, USA).

**Molecular identification of biting midges**

A 658-bp fragment of the mitochondrial cytochrome \( c \) oxidase subunit 1 gene ( \( \text{cox1} \) ) was amplified PCR, using the primers HCO2198 and LCO1490 [44]. One microliter template was added to a 10 μl reaction mix, containing 6.6 μl of Hotstar Taq Master Mix (Qiagen), 2.2 μl of molecular grade water (included in the Master Mix kit) and 0.6 μl of each 10 μM primer. The following cycling program was used: initial denaturation at 95 °C for 15 min, followed by 40 cycles of 30 s denaturation at 94 °C, 45 s annealing at 40 °C and 1 min extension at 72 °C, and final extension step for 10 min at 72 °C. Each PCR run included DNA of Culex quinquefasciatus Say, 1823 (positive control) and ultrapure water (negative control). All amplicons were visualised on 2% agarose gels and PCR products sequenced with LGC Genomics (Berlin, Germany).
Molecular identification of Culicoides hosts

Hosts were identified using two PCR protocols targeting the cytochrome b (cytb) and 16S rRNA gene fragment [45–47]. Both protocols were described in detail in a previous study by Börstler et al. [32]. If the amplification with the first pair of primers failed [45, 46], another PCR was applied using the second pair of primers [47]. The same applied to potential mixed blood meals as indicated by double peaks at different positions in the sequence electropherograms. These samples were also analysed with both PCRs. As observed in our previous studies [32, 33], the PCR targeting the cytb gene fragment generally has a higher amplification rate for mammals, and the PCR targeting the 16S rRNA gene fragment a higher amplification rate for birds. We used the DNA of a mammal (African green monkey, Chlorocebus sabaues (L.)) and a bird (European blackbird, Turdus merula L.) as positive controls. The negative control was ultrapure water, which was included in each PCR run. These amplicons were also visualised and sequenced as described above.

Data analysis

Sequences were visualised and edited with Geneious version 9.1.7 (Biomatters, Auckland, New Zealand). The resulting sequences were submitted for species identification using the basic alignment search tool (BLAST) in the GenBank DNA sequence database (https://blast.ncbi.nlm.nih.gov/) and the Barcode of Life Database [48]. In order to rule out potential contamination, samples indicating human host DNA were repeated separately in an individual PCR reaction. Identity values for the Culicoides and host species generally ranged between 98 and 100%. Sequences with lower identity values were repeated. One exception was the newly described haplotype of C. punctatus (Meigen, 1804), which showed identity values between 96 and 97%. In addition, information on the fauna of the DDBR were used to interpret the identity values for uncorrected genetic distances of approximately 4% to C. punctatus (Fig. 3). A distinct haplotype of C. punctatus (designated as C. punctatus P) was identified in almost half (n = 207, 45.5%) of the 454 C. punctatus specimens analysed. These clustered within a separate monophyletic clade showing a genetic distance of approximately 4% to C. punctatus (Fig. 3). For the unknown Culicoides we could not find any similar sequences in the databases. This group of specimens showed a divergence of 15.6–16.3% from the closest identified Culicoides species (data not shown). The sequences of these specimens had a high similarity with each other and clustered with C. kibunensis in a monophyletic clade (Fig. 3).

Results

Molecular identification of biting midges

Sequencing a fragment of the cox1 gene allowed the molecular identification of 1134 (89.7%) of the analysed midges (Table 1). Five species were identified for engorged biting midges: C. griseidorsum Kieffer, 1918; C. kibunensis Tokunaga, 1937; C. punctatus; C. rie thi Kieffer 1914; and C. submaritimus Tokunaga & Murachi, 1959. Culicoides subfasciipennis Kieffer, 1919/C. pallidicornis Tokunaga & Murachi, 1959 were not differentiated to the species level. Furthermore, 39 sequences (3.1% of the analysed specimens) could not be identified to species level by comparison with other Culicoides sequences available on GenBank. The sequences of these specimens had a high similarity indicative of belonging to the same species and represent the seventh taxon hereafter referred to as “unknown Culicoides”. The eighth taxon detected was C. puncticollis (Becker, 1903), only present in the non-engorged fed biting midges selected for morphological identification. Four of the seven detected engorged species were confirmed by morphology: C. griseidorsum; C. kibunensis; C. rie thi; and C. punctatus. In contrast, engorged C. submaritimus and C. subfasciipennis/C. pallidicornis were identified solely by barcoding and were not found in the small set of unfed specimens. Culicoides puncticollis was identified by morphology and cox1 barcoding, but only from the same subset of 37 unfed specimens (Additional file 3: Figure S1). As the cox1 sequences are not suitable to differentiate between C. subfasciipennis and C. pallidicornis [51, 52], these specimens were classified as C. subfasciipennis/C. pallidicornis. The unknown Culicoides species had similar wing patterns to C. kibunensis (Fig. 2).

In order to perform a identity verification of the generated Culicoides cox1 sequences, we constructed a maximum likelihood phylogenetic tree including conspecific Culicoides and outgroup sequences (Fig. 3). A distinct haplotype of C. punctatus (designated as C. punctatus P) was identified in almost half (n = 207, 45.5%) of the 454 C. punctatus specimens analysed. These clustered within a separate monophyletic clade showing a genetic distance of approximately 4% to C. punctatus (Fig. 3). For the unknown Culicoides we could not find any similar sequences in the databases. This group of specimens showed a divergence of 15.6–16.3% from the closest identified Culicoides species (data not shown). The sequences of these specimens had a high similarity with each other and clustered with C. kibunensis in a monophyletic clade (Fig. 3).
Table 1  Frequency of detected hosts per *Culicoides* spp. with corresponding percentage collected in the Danube Delta Biosphere Reserve (Romania) during 2017

| Host Information | *C. griseidorsum* n (%) | *C. kibunensis* n (%) | *C. punctatus* n (%) | *C. punctatus P* n (%) | *C. niethi* n (%) | *C. subalpiscipennis/C. pallidicornis* n (%) | *C. submaritimus* n (%) | Unknown *Culicoides* sp. n (%) | Host information without *Culicoides* identification n (%) | Total n (%) |
|------------------|-------------------------|-----------------------|----------------------|-------------------------|------------------|---------------------------------------------|-------------------------|---------------------------------|---------------------------------|------------|
| Mammals (n = 1064, 92%) |                         |                       |                      |                         |                  |                                             |                         |                                 |                                  |            |
| *Bos taurus* L. | 170 (63.9)              | 4 (5.2)               | 207 (85.9)           | 163 (81.1)              | 8 (80.0)         | 188 (83.2)                                  | 1 (0.4)                 | 2 (11.1)                        | 74 (68.5)                       | 817 (70.7) |
| *Bubalus bubalis* (Kerr) |                     |                       |                      |                         |                  |                                             |                         |                                 |                                  | 2 (0.2)    |
| *Capra hircus* L. | 46 (17.3)               | 1 (1.3)               | 3 (1.2)              | 1 (0.5)                 | 1 (10.0)         | 1 (0.4)                                     |                         |                                 |                                  | 53 (46)    |
| *Capreolus capreolus* (L.) |                    |                       |                      |                         |                  |                                             |                         |                                 |                                  | 1 (0.1)    |
| *Equus caballus* L. | 15 (5.6)                | 9 (3.7)               | 3 (1.5)              | 1 (10.0)                | 4 (1.8)          | 1 (0.4)                                     | 5 (4.6)                 | 10 (9.3)                       | 43 (37)                         | 38 (33)    |
| *Felis catus* L. | 1 (1.3)                 |                       |                      |                         |                  |                                             | 1 (0.4)                 |                                 |                                  | 2 (0.2)    |
| *Homo sapiens* L. | 3 (1.1)                 | 10 (13.0)             | 5 (2.1)              | 4 (2.0)                 | 4 (1.8)          | 5 (5.6)                                     | 2 (1.1)                 | 10 (9.3)                       | 43 (37)                         | 3 (0.2)    |
| *Sus scrofa* L. | 28 (10.5)               | 3 (3.9)               | 16 (6.6)             | 29 (14.4)               | 22 (9.7)         |                                            |                         |                                 |                                  | 101 (8.7) |
| Birds (n = 92, 8%) |                         |                       |                      |                         |                  |                                             |                         |                                 |                                  |            |
| *Acrocephalus arundinaceus* (L.) |                  | 1 (1.3)               |                       |                         |                  |                                             | 1 (0.4)                 |                                 |                                  | 2 (0.2)    |
| *Acrocephalus scirpaceus* (Hermann) |               |                       |                      |                         |                  |                                             |                         |                                 |                                  | 13 (11)   |
| *Ardea cinerea* L. | 1 (1.3)                 |                       |                      |                         |                  |                                             |                         |                                 |                                  | 1 (0.1)    |
| *Ardea purpurea* L. | 6 (7.8)                 |                       |                      |                         |                  |                                             |                         |                                 |                                  | 7 (0.8)    |
| *Columba palumbus* L. |                     |                       |                      |                         |                  |                                             |                         |                                 |                                  | 2 (0.2)    |
| *Coracias garrulus* L. |                     |                       |                      |                         |                  |                                             |                         |                                 |                                  | 1 (0.1)    |
| *Corvus corone* L. | 6 (7.8)                 | 3 (16.7)              | 3 (3.3)              | 6 (5.6)                 | 16 (1.1)         |                                             |                         |                                 |                                  | 6 (0.5)    |
| *Emberiza schoeniclus* (L) |                |                       |                      |                         |                  |                                             |                         |                                 |                                  | 1 (0.1)    |
| *Falco tinnunculus* L. |                    |                       |                      |                         |                  |                                             |                         |                                 |                                  | 2 (0.2)    |
| *Gallinula chloropus* (L) |                |                       |                      |                         |                  |                                             |                         |                                 |                                  | 3 (0.3)    |
| *Gallus gallus* (Gmelin) |                   |                       |                      |                         |                  |                                             |                         |                                 |                                  | 6 (0.5)    |
| *Hirundo rustica* L. | 2 (0.8)                 | 2 (2.6)               |                      |                         |                  |                                             |                         |                                 |                                  | 2 (0.2)    |
Table 1 (continued)

| Host                          | C. griseidorsum n (%) | C. kibunensis n (%) | C. punctatus P n (%) | C. punctatus n (%) | C. riethi n (%) | C. subfuscipennis/C. pallidicornis n (%) | C. submoranitus n (%) | Unknown Culicoides sp. n (%) | Host information without Culicoides identification n (%) | Total n (%) |
|-------------------------------|-----------------------|---------------------|----------------------|-------------------|----------------|----------------------------------------|----------------------|--------------------------------|---------------------------------------------------------|------------|
| Meleagris gallopavo L.        |                       |                     |                      |                   |                |                                        |                      |                                | 1 (0.1)                                                | 1 (0.1)   |
| Motacilla alba L.             | 1 (1.3)               |                     |                      |                   |                |                                        |                      |                                | 1 (0.1)                                                | 1 (0.1)   |
| Nycticorax nycticorax (L.)    | 1 (1.3)               | 1 (0.4)             |                      |                   |                |                                        |                      | 1 (5.6)                                      | 3 (0.3)                                                | 3 (0.3)    |
| Parus major L.                | 1 (1.3)               |                     |                      |                   |                |                                        |                      |                                | 3 (2.8)                                                | 4 (0.3)    |
| Passer montanus (L.)          | 1 (1.3)               |                     |                      |                   |                |                                        |                      |                                | 1 (0.1)                                                | 1 (0.1)   |
| Phalacrocorax carbo (L.)      |                       |                     |                      |                   |                |                                        |                      |                                | 2 (1.9)                                                | 2 (0.2)    |
| Sternotopelia decollata       |                       |                     |                      |                   |                |                                        |                      |                                | 2 (1.1)                                                | 2 (0.2)    |
| Streptopelia decollata        |                       | 1 (1.3)             |                      |                   |                |                                        |                      |                                | 1 (0.1)                                                | 1 (0.1)   |
| Strix aluco L.                | 1 (1.3)               |                     |                      |                   |                |                                        |                      |                                | 1 (0.1)                                                | 1 (0.1)   |
| Sylvia borin (Boddart)        | 1 (0.4)               | 4 (5.2)             |                      |                   |                |                                        |                      |                                | 5 (0.4)                                                | 5 (0.4)    |
| Tito alba (Scopoli)           | 1 (1.3)               |                     |                      |                   |                |                                        |                      |                                | 1 (0.1)                                                | 1 (0.1)   |
| Asio otus (L.)                | 2 (2.6)               |                     |                      |                   |                |                                        |                      |                                | 2 (0.2)                                                | 2 (0.2)    |
| Tito alba/Asio otus           | 2 (2.6)               |                     |                      |                   |                |                                        |                      |                                | 1 (0.9)                                                | 3 (0.3)    |
| Culicoides specimens without host identification | 11 | 26 | 7 | 6 | 2 | 19 | 1 | 21 |
| Total biting midge specimens | 276$^a$ | 102$^c$ | 248 | 207 | 12 | 242$^c$ | 8$^d$ | 39$^e$ |

$^a$ Including one mixed blood meal: Bos taurus + Gallus galus

$^b$ Including one mixed blood meal: Sus scrofa + Homo sapiens

$^c$ Including three mixed blood meals: Bos taurus + Canis lupus familiaris; Sus scrofa + Acrocephalus arundinaceus; Bos taurus + Nycticorax nycticorax

$^d$ Including two mixed blood meals: Corvus corone + Homo sapiens

$^e$ Including one mixed blood meal: Equus caballus + Hirundo rustica
Fig. 2  Two wing pictures for the unknown *Culicoides* species collected in the Danube Delta Biosphere Reserve (Romania) during 2017.

Fig. 3  Maximum likelihood phylogenetic tree of *cox1* sequences for *Culicoides* species collected in the Danube Delta Biosphere Reserve (Romania) during 2017. Silhouettes indicate observed host-feeding patterns regarding the relative frequencies of mammalian and avian hosts. The tree was inferred using an HKY + G model (1000 bootstrap replicates) and rooted with *Forcipomyia* sp. and *Culex quinquefasciatus*. Branch support values of ≥ 50% are displayed and GenBank accession numbers of sequences shown on the branch tips.
Culicoides punctatus \((n=455, 36.0\%\) of all analysed specimens), C. griseidorsum \((n=276, 21.8\%\), C. subfasciipennis//C. pallidicornis \((n=242, 19.1\%\) and C. kibunensis \((n=102, 8.1\%\) were the most frequent taxa identified (Table 1). Blood-meal identification was not possible for 93 specimens due to failed PCR amplification. In addition, eight mixed blood meals were detected. With the exception of C. punctatus \((n=455)\) and C. riethi \((n=12)\), mixed blood meals where found for engorged specimens of all five Culicoides spp. Two Culicoides specimens contained blood from two mammalian hosts, while the other six specimens had mixed blood meals from a bird and a mammal.

A total of 33 vertebrate species were identified including nine species of mammals (27.3\%) and 24 species of birds (72.7\%) (Table 1). Mammals dominated the host spectrum \((n=1064, 92.0\%\) of all 1156 identified blood sources). Cattle \((Bos taurus)\) was the most abundant species \((n=817, 70.7\%\), followed by wild boar \((n=101, 8.7\%\). Other mammalian hosts were each found at a rate below 5\%. Birds amounted to 8\% of all the identified hosts with the Eurasian reed warbler \((Acrocephalus scirpaceus; n=13, 1.12\%)\) and the carrion crow \((Corvus corone; n=16, 1.38\%)\) as most frequent.

With the exception of C. riethi \((n=12)\), at least one avian host was detected for all Culicoides spp. Birds dominated the blood-meal sources of C. kibunensis and the unknown Culicoides sp. \((68.8\%\) and 72.2\% of the detected hosts, respectively) (Table 1). Culicoides kibunensis had the highest diversity of hosts, with seven \((77.8\%)\) of the nine mammalian hosts and 18 \((75\%)\) of 24 species of avian hosts. Furthermore, humans were the most frequent mammalian host for this species \((n=10, 13.0\%\) of all identified hosts). In contrast, the three most frequent Culicoides spp. \((C. griseidorsum, C. punctatus and C. subfasciipennis//C. pallidicornis)\) showed high proportions of cattle \((between 63.9 and 85.9\%\) of all identified blood sources per taxon). The second most frequent hosts were goat \((Capra hircus)\) for C. griseidorsum \((17.3\%)\) and wild boar for C. punctatus \((6.6\%)\), C. punctatus P \((14.4\%)\) and C. subfasciipennis//C. pallidicornis \((9.7\%)\) (Table 1). No differences were observed between C. punctatus and its distinct haplotype C. punctatus P. Furthermore, for C. submaritimus \((n=8)\) only blood meals from humans \((n=5)\), carrion crows \((n=3)\) and cattle \((n=1)\) were detected.

**Discussion**

The relevance of Culicoides spp. as important vectors of pathogens is well known. Thus, information about their diversity and host-feeding patterns is crucial to understand parasite-host interactions and the ecology of associated pathogens [30]. DNA barcoding is an important tool in biodiversity studies [53–57]. Thereby, barcoding also helped to identify cryptic and new Culicoides species [58–60]. In this study, successful sequencing of 1040 engorged insects demonstrated that barcoding is a useful tool for both, Culicoides and host identification. However, it must be considered that the different genetic markers can have pitfalls and do not necessarily reflect morphological differences [56, 61], i.e. using a single marker might be insufficient for an accurate identification of species.

A total of seven Culicoides species-level taxa were detected for the four sites in the DDBR. In the phylogenetic tree, specimens of the same taxon clustered in well-supported terminal clades. The only exception was C. subfasciipennis//C. pallidicornis. The separation between these two species is based on a variable light spot on the wing’s anal cell of C. subfasciipennis [14]. However, the analysis indicated no sequence differences of the cox1 gene. The discriminatory characters on the wing might be unreliable and further studies are required to clarify the status of both species [51, 52].

Culicoides griseidorsum, C. puncticollis and C. submaritimus were recorded for the first time in Romania, increasing the number of known Culicoides species for the country to 49 species [25]. Culicoides submaritimus has been considered a synonym of C. maritimus Toku-naga, 1940 by some authors [62, 63], while recent studies treated C. submaritimus as a distinct species [14, 64]. In the present study, C. submaritimus was identified by its similarity with cox1 sequences from Turkey, which are the only sequences available on GenBank for this species, while no cox1 sequences were available for C. maritimus. Neither C. submaritimus, nor C. maritimus are included in the inventory of Culicoides biting midges of Romania [25], although more recent studies include the country in the distribution of C. maritimus [14, 65].

The observed genetic variation for the analysed C. punctatus in two distinct clades is within intraspecific boundaries [59]. Such sibling species may vary in their vectorial capacity [66], e.g. vector competence...
or host-feeding patterns of members in the *Anopheles gambiense* complex. However, we did not detect differences in the host-feeding patterns between either taxa. Furthermore, the specimens clustering within the clade designated as "unknown *Culicoides*" showed genetic distances of 15.6–16.3% from the closest described species. These distances are similar to those observed between the other *Culicoides* species in our study. Comparable distances were found in other *Culicoides* spp. [67, 68] or mosquitoes [69], indicating that these specimens belong to a separate new species or a species without reference sequences in molecular libraries.

The overall host spectrum covered species expected for the DDBR, including livestock species like buffalo (*Bubalis bubalis*). Therefore, most of the analysed *Culicoides* spp. had a broad host-feeding range. Only mammalian hosts were detected for *C. riethi*, but the small sample size of only 12 engorged specimens does not allow an accurate conclusion on the species’ host-feeding pattern. Both, mammalian and avian hosts were detected for all other biting midge taxa to various extents. The broad host choice matches previous studies, which found similar results for different *Culicoides* spp. [70, 71]. Humans and carrion crow were the only hosts of *C. submaritimus* (*n* = 8). Cattle, wild boar or goat dominated the hosts of the three most frequent *Culicoides* taxa (*C. punctatus*, *C. subfasciipennis*/*C. pallidicornis* and *C. griseidorsum*). The high frequency of cattle probably relates to the large number of free-roaming cattle available in the DDBR and their large body mass [72]. However, as observed before [41, 67, 73, 74], despite this distinct dominance of mammalian hosts, different avian hosts were detected for the three *Culicoides* taxa.

*Culicoides kibunensis* is considered predominantly ornithophilic [37, 38, 75, 76]. With 18 species of birds and seven species of mammals, this vector of avian malaria [37, 38] showed the highest overall host diversity. The wide range of bird species is not surprising, considering the diversity of this vertebrate group in the DDBR. Nevertheless, the observed generalist host-feeding pattern including humans match previous studies [34, 37, 38]. Interestingly, the unknown *Culicoides* species showed a similar host-feeding pattern as *C. kibunensis*, with which it formed a monophyletic clade in the phylogenetic tree. These observations support the hypothesis of a positive correlation between biting midge phylogenetic relatedness and their feeding behaviour [40, 77]. In contrast, other studies speculated that such similarities in host-feeding patterns are not necessarily driven by phylogenetic relatedness, but might be the result of other factors (e.g. body size-driven host choice due to larger emissions of CO$_2$ or volatile compounds) [71].

Host availability probably has a significant impact on the observed host-feeding patterns of *Culicoides* spp. Although no quantitative information on the host community is available, the prevalence of humans and domestic animals at Dunărea Veche and Lake Roșuleț is known. Humans, dogs and cats had relative low abundance at both sites compared to birds or free-ranging cattle and horses. Nevertheless, humans, dogs or cats were detected as hosts for all analysed *Culicoides* species. Thus, caution regarding the distribution of biting midges and the potential host has to be considered when interpreting host-feeding patterns of *Culicoides*. For example, a high proportion of *C. griseidorsum* were found to have fed on goats, but this host was widely available at Letea, where most of this species were collected (Additional file 4: Table S2, Additional file 5: Table S3).

Information on the host-feeding patterns can be also used to estimate dispersal distances of *Culicoides* spp. [77]. Biting midges from the sampling site Dunărea Veche were engorged with blood from buffalo and goat. These hosts are only available in the nearest village more than 4 km from the trapping site, which is in the range of a previous study on *Culicoides* [78]. Maximum dispersal distances of more than 3 km over one night were recorded regularly. Winds over the delta’s flat landscape might favour passive dispersal [79–82]. Thereby, besides active midge movement, wind dispersal is considered an important mode of long-distance dispersal for *Culicoides*-borne pathogens [83–85].

### Conclusions

The broad host range of different mammalian and avian species indicates that most of the analysed *Culicoides* species in the DDBR are potential bridge vectors. However, the actual vector competence of these species is largely unknown. Of the dominant *Culicoides* species analysed, *C. punctatus* was previously indicated as a potential vector of BTV and SBV [86, 87]. Free roaming cattle, the most abundant and most frequently detected hosts in the region, could have an important role in amplification and spread of pathogens between wild ruminants and livestock [88]. At the same time, the new records of biting midge taxa for the country presented here and the detection of a potentially unknown *Culicoides* taxon highlight the lack of knowledge regarding the biting midge species and their genetic diversity in Europe.
Supplementary information

Supplemental information accompanies this paper at https://doi.org/10.1186/s13071-020-3938-1.

Additional file 1: Text S1. Description of the sampling sites with information on vegetation, surrounding environment and available hosts.

Additional file 2: Table S1. Accession numbers of Culicoides spp., Forcipomyia spp. and Culex quinquefasciatus used for phylogenetic analysis.

Additional file 3: Figure S1. Wing patterns for C. punctatus, C. punctatus P, C. kibunensis, C. puncticollis, C. nethi and C. gisręsoridum collected in this study.

Additional file 4: Table S2. Overview of the Culicoides species per sampling site.

Additional file 5: Table S3. Overview of the frequency of each molecularly identified Culicoides host species per sampling site.

Abbreviations
DDBR: Danube delta biosphere reserve; DNA: Deoxyribonucleic acid; BTV: Bluetongue virus; SBV: Schmallenberg virus; BG trap: Biogents sentinel trap; PCR: Polymerase chain reaction.

Acknowledgements
We would like to thank to Vasile Suhov and the residents of Sulina and Letea, who granted access to their properties. In addition, we are very grateful to Dr Andreas Krüger for his support and fruitful discussion. In addition, we thank Patricia Iftene for help during the field work, Daniel Truchado for his help with literature research.

Authors’ contributions
AT, HJ, MS, JSC, DC and RL conceived and designed the study. AT, HJ and JS collected the data. AT, HJ, JSC, DC and RL analyzed the data. AT, DC and RL drafted the manuscript. All authors read and approved the final manuscript.

Funding
Not applicable.

Availability of data and materials
The data supporting the conclusions of this article are included within the article and its additional files.

Ethics approval and consent to participate
The DDBR administration approved all research activities for trapping at specific study sites (9/19.04.2017, 5627/ARRBDD/13.04.2017).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1 WHO Collaborating Centre for Arbovirus and Hemorrhagic Fever Reference and Research, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany. 2 University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania. 3 Faculty of Mathematics, Informatics and Natural Sciences, Universität Hamburg, Hamburg, Germany.

Received: 24 September 2019 Accepted: 3 February 2020
Published online: 17 February 2020

References
1. Linkery J. Biting midges as vectors of nonviral animal pathogens. J Med Entomol. 1985;22:589–99.
2. Chagas CRF, Bukauskaite D, Igunas M, Iezhova T, Valkiunas G. A new blood parasite of leaf warblers: molecular characterization, phylogenetic relationships, description and identification of vectors. Parasit Vectors. 2018;11:538.
3. Svobodová M, Dolník OV, Ivan C, Rádvová J. Biting midges (Ceratopogonidae) as vectors of avian trypanosomes. Parasit Vectors. 2017;10:224.
4. Debrah LB, Naush N, Opoku OS, Owusu W, Mubarik Y, Berko DA, et al. Epidemiology of Mansonella perstans in the middle belt of Ghana. Parasit Vectors. 2017;10:15.
5. Borkent A. The biting midges, the Ceratopogonidae (Diptera). In: Marquardt WG, editor. Biology of disease vectors. 2nd ed. Burlington MA: Elsevier Academic Press; 2005. p. 113–6.
6. da Rosa JF, de Souza WM, de Paula Pinheiro F, Figueiredo ML, Cardoso JF, Acrani GO, et al. Oropouche virus: clinical, epidemiological, and molecular aspects of a neglected orthobunyavirus. Am J Trop Med Hyg. 2017;96:1019–30.
7. Sick F, Beer M, Kampen H, Wernike K. Culicoides biting midges—underestimated vectors for arboviruses of public health and veterinary importance. Viruses. 2019;11:376.
8. Pinior B, Firth CL, Loitsch A, Stockreiter S, Hutter R, Richter V, et al. Cost distribution of bluetongue surveillance and vaccination programmes in Austria and Switzerland (2007–2016). Vet Rec. 2018;182:527.
9. Maan S, Maan NS, Ross-smith N, Batten CA, Shaw AE, Anthony SJ, et al. Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. Virology. 2008;377:308–18.
10. Foxi C, Delrio G, Falchi G, Marche MG, Satta G, Ruiu L. Role of different Culicoides vectors (Diptera: Ceratopogonidae) in bluetongue virus transmission and overwintering in Sardinia (Italy). Parasit Vectors. 2016;9:440.
11. Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PPC, Baylis M. Climate change and the recent emergence of bluetongue in Europe. Nat Rev Microbiol. 2005;3:171–81.
12. Nolan DV, Carpenter S, Barber J, Mellor PS, Dallas JF, Mordue AJ, et al. Rapid diagnostic PCR assays for members of the Culicoides obsoletus and Culicoides pulicaris species complexes, implicated vectors of bluetongue virus in Europe. Vet Microbiol. 2007;124:82–94.
13. Augot D, Sauvage F, Jouret D, Simphal E, Veillie M, Couloux A, et al. Discrimination of Culicoides obsoletus and Culicoides sculptus, potential bluetongue vectors, by morphometric and mitochondrial cytochrome oxidase subunit I analysis. Infect Genet Evol. 2010;10:629–37.
14. Mathieu B, Cèrette-Sossah C, Garros C, Chavernac D, Balenghieng T, Carpenter S, et al. Development and validation of iFC: an interactive identification key for Culicoides (Diptera: Ceratopogonidae) females from the Western Palaearctic region. Parasit Vectors. 2012;5:137.
15. Cuéllar AC, Kjaer LJ, Kirkeby C, Skovgard H, Nielsen SA, Stockmarr A, et al. Spatial and temporal variation in the abundance of Culicoides biting midges (Diptera: Ceratopogonidae) in nine European countries. Parasit Vectors. 2018;11:12.
16. Lühken R, Kiel E, Steinkühle S, Fladung R. Tsetse and Glossina: a comprehensive guide to the tsetse flies and their disease vectors. Cambridge: Cambridge University Press; 2007. p. 1–376.
17. Garros C, Vennet E, Rossi S, Balinghien T. Adaptation of a species-specific multiplex PCR assay for the identification of blood meal source in Culicoides (Ceratopogonidae: Diptera): applications on Palaearctic biting midge species, vectors of orbiviruses. Infect Genet Evol. 2011;11:1103–10.
18. Carpenter S, Veronesi E, Mullens B, Venter G. Vector competence of Culicoides for arboviruses: three major periods of research, their influence on current studies and future directions. Rev Sci Tech. 2015;34:97–112.
19. Barber J, Harrup LE, Silk R, Veronesi E, Gubbins S, Bachanek-Bankowska K, et al. Blood-feeding, susceptibility to infection with Schmallenberg virus and phylogeogenetics of Culicoides (Diptera: Ceratopogonidae) from the United Kingdom. Parasit Vectors. 2018;11:316.
20. Pagès N, Talaveras S, Verdún M, Pujol N, Valle M, Bensaid A, et al. Schmallenberg virus detection in Culicoides biting midges in Spain: first laboratory evidence for highly efficient infection of Culicoides of the obsolatus complex and Culicoides imicola. Transbound Emerg Dis. 2018;65:e1–6.
21. Kynakis CS, Billinis C, Papadopoulos E, Vasilieou NG, Athanasiou LV, Pthenakis GC. Bluetongue in small ruminants: an opinionated review, with a brief appraisal of the 2014 outbreak of the disease in Greece and the south-east Europe. Vet Microbiol. 2015;181:66–74.
22. Stojmanovski Z, Tabakovski B. Spatio-temporal characteristics of the bluetongue epizooty in the Balkan Peninsula from 2014 to February 2015. Maced Vet Rev. 2018;41:65–72.

23. Trilbaša EM, Popescu D, Badea C, Hora FS, Dărăbău G. A report regarding the first occurrence of bluetongue in Romania, 2014. Scientific Works Series C. Vet Med. 2015;61:277–80.

24. Damn argentescu A. New species of Ceratopogonidae (Diptera) for the Romanian fauna. Studii si cercetari de biologie, Seria Zoologie. 1972;24:423–32.

25. Damn argentescu A. Familia Ceratopogonidae, Genus Culicoides: Fauna româniei, insecta, fasc 14 diptera, vol. 11. Bukuresti: Editura Academiei Române, 2000.

26. Trilbaša EM, Dărăbău G. Preliminary studies on dynamics of Culicoides spp. in western Romania in conjuncture with some environmental factors. Parasit Vectors. 2014;7(Suppl. 1):07.

27. Dărăbău G, Trilbaša E, Oprescu I, Moraor S, Mederre N, Ilie M, Sujic T, Imre M. The abundance of Culicoides (Diptera: Ceratopogonidae) in Timiş county. Scient Works Med Vet. 2017;50:1689–99.

28. Ionță M, Mitrea IL, Buzatu MC, Dascălu L, Ionescu A. Seasonal dynamics of haematophagous arthropod populations (ticks and Culicoides spp.)—vectors of pathogens in animals and humans, in different areas of Romania. Scientiс Works Med Vet. 2009;52:629–36.

29. Ilie A, Șerban C, Imre M, Sorescu D, Ilie M, Imre K, et al. A survey (or presence, dynamics, prevalence) of Culicoides (Diptera: Ceratopogonidae) in Gorj county, Romania preliminary results of entomological surveillance for bluetongue. Scient Works Med Vet. 2013;46:5–9.

30. Kent RJ. Molecular methods for arthropod bloodmeal identification and applications to ecological and vector-borne disease studies. Mol Ecol Resour. 2009;9:4–18.

31. Bitome-Essono PY, Ollomo B, Arnathau C, Durand P, Mokoudoum ND, Kent RJ. Molecular methods for arthropod bloodmeal identification and application in epidemiological studies. Mol Ecol Notes. 2007;7:355–64.

32. Börstler J, Jöst H, Garms R, Krüger A, Tannich E, Becker N, et al. Host-feeding patterns of Culicoides (Diptera: Ceratopogonidae) vectors, and avian hosts of biting midges in Iran. Iran J Biomed. 2015;31:105–16.

33. Shahhosseini N, Friedrich J, Moosa-Kazemi SH, Sedaghat MM, Kayedi MH, Hedayati MS, Jafari S, et al. The role of Culicoides sonorensis (Diptera: Ceratopogonidae) as a vector of Cache Valley virus in Iran. Int J Med Microbiol. 2018;318:426–32.

34. Hadj-Henni L, De Meulemeester T, Depaquit J, Noël P, Germain A, Helder L. Bloodmeal data of Culicoides (Diptera: Ceratopogonidae) from Scandinavia. Parasit Vectors. 2017;10:279.

35. Talavera S, Muñoz-Muñoz F, Verdun M, Pujol N, Figuerola J, Soriguer R, et al. Bloodmeal feeding patterns of potential arbovirus vectors of the genus Culicoides targeting ectothermic hosts. Ann Trop Med Hyg. 2008;79:980–15.

36. Kent RJ, Kent C, Boyer J. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9.

37. Burkett-Cadena ND, Graham SP, Hassan HK, Guyer C, Eubanks MD, Katholi CR, et al. Blood feeding patterns of potential arbovirus vectors of the genus Culicoides targeting ectothermic hosts. Ann Trop Med Hyg. 2008;79:980–15.

38. Teixeira JP, Bradley G, Barlaz MA, Graham SP, Grass CC, et al. DNA barcoding of British mosquitoes (Diptera, Culicidae) to support species identification, discovery of cryptic genetic diversity and monitoring invasive species. BioControl. 2010;55:339–46.

39. Talavera S, Muñoz-Muñoz F, Verdun M, Pujol N, Figuerola J, Soriguer R, et al. Bloodmeal feeding patterns of potential arbovirus vectors of the genus Culicoides targeting ectothermic hosts. Ann Trop Med Hyg. 2008;79:980–15.

40. Kent RJ, Kent C, Boyer J. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9.

41. Burkett-Cadena ND, Graham SP, Hassan HK, Guyer C, Eubanks MD, Katholi CR, et al. Blood feeding patterns of potential arbovirus vectors of the genus Culicoides targeting ectothermic hosts. Ann Trop Med Hyg. 2008;79:980–15.

42. Kent RJ, Kent C, Boyer J. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9.

43. Burkett-Cadena ND, Graham SP, Hassan HK, Guyer C, Eubanks MD, Katholi CR, et al. Blood feeding patterns of potential arbovirus vectors of the genus Culicoides targeting ectothermic hosts. Ann Trop Med Hyg. 2008;79:980–15.

44. Kent RJ, Kent C, Boyer J. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9.

45. Burkett-Cadena ND, Graham SP, Hassan HK, Guyer C, Eubanks MD, Katholi CR, et al. Blood feeding patterns of potential arbovirus vectors of the genus Culicoides targeting ectothermic hosts. Ann Trop Med Hyg. 2008;79:980–15.
67. Pettersson E, Bensch S, Ander M, Chrico J, Sigvald R, Ignell R. Molecular identification of bloodmeals and species composition in Culicoides biting midges. Med Vet Entomol. 2013;27:104–12.
68. Ander M, Troell K, Chrico J. Barcoding of biting midges in the genus Culicoides: a tool for species determination. Med Vet Entomol. 2013;27:323–31.
69. Chan A, Chiang LP, Hapuarachchi HC, Tan CH, Pang SC, Lee R, et al. DNA barcoding: complementing morphological identification of mosquito species in Singapore. Parasit Vectors. 2014;7:569.
70. Tempelis CH, Nelson RL. Blood-feeding patterns of midges of the Culicoides variipennis complex in Kern county, California. J Med Entomol. 1971;8:532–4.
71. Hopken MW, Ryan BM, Huyvaert KP, Paigio AJ. Picky eaters are rare: DNA-based blood meal analysis of Culicoides (Diptera: Ceratopogonidae) species from the United States. Parasit Vectors. 2017;10:169.
72. Viennet E, Garros C, Gardès L, Rakotoarivony I, Allègre X, Lancelot R, et al. Host preferences of palaearctic Culicoides biting midges: implications for transmission of orbiviruses. Med Vet Entomol. 2013;27:253–66.
73. Lassen SB, Nielsen SA, Skovgaard H, Kristensen M. Molecular identification of bloodmeals from biting midges (Diptera: Ceratopogonidae: Culicoides Lattreille) in Denmark. Parasitol Res. 2011;108:823–9.
74. Calvo JH, Bertal B, Calvete C, Miranda MA, Estrada R, Lucientes J. Host feeding patterns of Culicoides species (Diptera: Ceratopogonidae) within the Picos de Europa National Park in northern Spain. Bull Entomol Res. 2012;102:692–7.
75. Černý O, Votýpka J, Svobodová M. Spatial feeding preferences of ornithophilic mosquitoes, blackflies and biting midges. Med Vet Entomol. 2011;25:104–8.
76. Ander M, Troell K, Chrico J. Barcoding of biting midges in the genus Culicoides
77. Augot D, Hadj‑Henni L, Strutz SE, Slama D, Millot C, Depaquit J, et al. Collection of wind‑borne haematophagous insects in the Torres Strait, Australia. Med Vet Entomol. 2003;17:102–9.
78. Conraths FJ, Gethmann JM, Staubach C, Mettenleiter TC, Beer M, Hoff‑mann B. Epidemiology of bluetongue virus serotype 8, Germany. Emerg Infect Dis. 2009;15:467–73.
79. Greenberg JA, DiMenna MA, Hanelt B, Hofkin BV. Analysis of post-blood meal flight distances in mosquitoes utilizing zoo animal blood meals. J Vector Ecol. 2012;37:83–9.
80. Sanders CJ, Selby R, Carpenter S, Reynolds DR. High‑altitude flight of Culicoides biting midges. Vet Rec. 2011;169:208.
81. Johansen CA, Farrow RA, Morrisen A, Foley P, Bellis G, Van Den Hurk AF, et al. Detection of the Schmallenberg virus in nulliparous Culicoides obsoletus scoticus/C. punctatus—the possibility of transovarial virus transmission in the Culicoides punctatus species complex in Kern county, California. J Med Entomol. 1971;8:532–4.
82. Kluiters G, Swales H, Baylis M. Local dispersal of palaearctic Culicoides biting midges estimated by mark‑release‑recapture. Parasit Vectors. 2015;8:86.
83. Ducheyeine E, De Deken R, Bécu S, Codina B, Nomikou K, Mangana‑Vougiaki O, et al. Quantifying the wind dispersal of Culicoides species in Greece and Bulgaria. Geospat Health. 2007;1:177.
84. Sedda L, Brown HE, Purse BV, Burgin L, Gistler J, Rogers DJ. A new algorithm quantifies the roles of wind and midge flight activity in the bluetongue epizootic in northwest Europe. Proc R Soc B. 2012;279:2354–62.
85. Burgin LE, Gistler J, Sanders C, Mellor PS, Gubbins S, Carpenter S. Investigating incursions of bluetongue virus using a model of long‑distance Culicoides biting midge dispersal: investigating bluetongue virus incursions. Transbound Emerg Dis. 2013;60:263–72.
86. Goffredo M, Catalani M, Federici V, Portanti O, Marini V, Mancini G, et al. Detection of Culicoides species in Singapore. Parasit Vectors. 2014;7:569.
87. Larska M, Lechowski L, Grochowska M, Zmudziński JF. Detection of the Schmallenberg virus in nulliparous Culicoides absoutus/scoticus complex and C. punctatus—the possibility of transovarial virus transmission in the midge population and of a new vector. Vet Microbiol. 2013;166:467–73.
88. At BMC, research is always in progress.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.