

Article

What Can Haemosporidian Lineages Found in *Culicoides* Biting Midges Tell Us about Their Feeding Preferences?

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Abstract: *Haemoproteus* (*Parahaemoproteus*) parasites are transmitted by *Culicoides* biting midges. However, the natural vectors of only six of the almost 180 recognized *Haemoproteus* species have been identified. The aim of this study was to investigate wild biting midges naturally infected with *Haemoproteus* and to understand the interaction network between *Culicoides* and *Haemoproteus* in Europe. *Culicoides* were collected with UV light traps from different sites in Lithuania. Parous females were morphologically identified based on their wings and heads. PCR-based methods were used to detect the *Haemoproteus* DNA, and salivary gland preparations were analyzed for the presence of sporozoites. Of the 580 *Culicoides* analyzed, 5.9% were positive for *Haemoproteus* DNA, and sporozoites were found in two of 11 sampled biting midge species: *Culicoides kibunensis* and *Culicoides segnis*. The interaction network revealed that *C. kibunensis* and *C. segnis* are frequently associated with several *Haemoproteus* lineages. On the other hand, some *Haemoproteus* lineages were found to interact with only one *Culicoides* species. This was the first report of *C. segnis* being a competent vector for *H. minutus* TURDUS2, *H. asymmetricus* TUPHI01, *H. majoris* PHSIB1, and *H. fringillae* CCF3; and of *C. kibunensis* being a competent vector for *H. belopoloskyi* HIICT1. *Culicoides segnis* and *C. kibunensis* are both important vectors of *Haemoproteus* parasites.

Keywords: biting midges; *Culicoides*; feeding preference; haemosporidian; *Haemoproteus*; host-parasite interactions; interaction network; natural vectors; natural infections; transmission



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1. Introduction

Haemoproteus (*Parahaemoproteus*) parasites are commonly found in birds, sometimes causing serious diseases and even leading to the high mortality of infected individuals [1]. They are transmitted by *Culicoides* biting midges; however, little is known about their natural vectors. There are almost 180 described species of *Haemoproteus* parasites [1], and more than 1400 species of *Culicoides* worldwide [2]; however, the natural vectors of *Haemoproteus* are known for only a small portion of parasite species [3–6]. Further, considering only studies combining the investigation of sporozoites in salivary gland preparations and using molecular tools to confirm parasite species and lineage, this number is even smaller [3–6].

Only 14 species of biting midges have been confirmed to be PCR-positive for *Haemoproteus* parasite DNA in Europe [3,5–12], which merely indicates that they had fed on infected birds, but not that the insect is a competent vector for the parasite. For this confirmation, it is necessary to prove the presence of sporozoites (the infective stage of haemosporidian parasites) in the insect salivary glands [13], which, in natural infections, was done for only six species of *Haemoproteus* (*H. pallidus* cytochrome *b* lineage PFC1, *H. parabelopoloskyi* SYAT02, *H. majoris* CCF5, *H. tartakovskiyi* HAWF1, *H. minutus* TURDUS2, and *H. asymmetricus* TUPHI01) and three species of *Culicoides* (*C. kibunensis*, *C. pictipennis*, and *C. segnis*) [3,5,6].

Additionally, many experimental studies have been conducted to investigate *Haemoproteus* development in *Culicoides* biting midges [4,14–16]. Even though they were able to prove that the parasite can develop in a certain *Culicoides* species, this does not mean that it is what occurs in nature. If the *Culicoides* species does not naturally feed upon the infected vertebrate host, it will not acquire the parasite, or transmit it. For example, the majority of these experimental studies have been conducted with *C. impunctatus* and *C. nubeculosus* [4], but so far, these two biting midge species have not been proven to be natural vectors of *Haemoproteus* parasites [3,5,6], probably because they are mammalophilic biting midge species, feeding on birds only opportunistically [17–19].

Understanding the transmission of vector-borne diseases is not so straightforward, given that the insect must first be interested in biting an infected bird, and then survive until the parasite completes its development, which means that the infective stages of the parasite are present in the salivary glands of the insect (in the case of haemosporidians), and finally feeds on a susceptible host [20]. However, many other factors are involved in this interaction, such as the feeding preference, reproductive biology, and attack rates of insects, as well as the defensive behavior, age, species, and health of the hosts [20]. It is also necessary to mention that parasites are evolutionarily adapted to evade the host and vector immune systems, replicate in host tissues, and surpass infection and transmission barriers in the insect body, which can also interfere in the transmission rates of vector-borne diseases [21].

This shows how complex host-parasite-vector relationships can be and how important it is to focus on studies targeting these relationships to understand the natural transmission of avian blood parasites. This study aimed to demonstrate the vector competence of *Culicoides* biting midges naturally infected with *Haemoproteus* parasites and thereby confirm their role as natural vectors of these avian parasites. Additionally, we also compared the interaction network between *Culicoides* biting midges and *Haemoproteus* lineages in Europe to better understand the parasite-vector relationship. For this, we considered all studies published to date in Europe reporting *Culicoides* biting midges that were PCR-positive for *Haemoproteus* DNA.

2. Materials and Methods

2.1. Biting Midges Collection

Biting midges were collected between June and September 2021 using UV LED-light traps (BG-Pro All-In-One Biogents AG) in different regions of Lithuania: Verkiai Regional Park (54°45′00″ N, 25°17′00″ E), Vilnius University Botanical Garden (54°44′12.5″ N 25°24′16.4″ E), Puvočiai (54°06′52.2″ N 24°18′17.6″ E), Ventės Ragas (55°20′28.1″ N, 21°11′25.3″ E) and its surroundings (55°23′57.5″ N 21°14′14.8″ E and 55°26′12.0″ N 21°16′04.6″ E) (Figure 1). The study sites were chosen due to their proximity to water bodies (such as lagoons and rivers), as well as their soil humidity, closed woods, and lack of wind. The traps were hung 6–7 h before sunset and removed 4–5 h after sunrise. Biting midges were collected into a small pot containing water with a drop of liquid soap.

Samples were transported to the laboratory right after collection for processing. The fresh material was investigated under a binocular stereo microscope, and female *Culicoides* biting midges with burgundy pigment (which indicates that at least one gonotrophic cycle occurred, meaning that there is a greater chance that the insect had, at least, one blood meal [22] and thus, was more likely to yield sporozoites of haemosporidian parasites) were dissected for salivary gland preparations.

2.2. Biting Midges Dissection, Identification, and Microscopic Examination of Salivary Gland Preparations

During dissection, each insect was placed in a drop of 0.9% saline solution on a glass slide. The head and wings were removed and transferred to a new glass slide containing a small drop of Euparal, covered with a cover slide, and dried at room temperature for two

months or in an incubator at 60 °C for one week. These permanent preparations were then used for morphological identification of dissected *Culicoides* [23–25].



Figure 1. Collection sites in Lithuania: Verkių Regional Park (1), Vilnius University Botanical Garden (2), Puvociai (3 and 4), Ventės Ragas (5), and its surroundings (6). Produced with Google Earth Pro v. 7.3.6.9277.

The salivary glands are in the anterior/upper part of the insect's thorax [13], which was gently crushed using dissecting needles to prepare a small thin smear [26]. To avoid contamination, all dissecting needles were disinfected in fire after each dissection. The salivary gland preparations were air dried, fixed with a drop of absolute methanol, and stained with a 4% Giemsa solution [13,26]. Remnants of dissected biting midges were stored in 96% alcohol for PCR-based analysis.

All salivary gland preparations of insects that were PCR-positive for *Haemoproteus* parasite DNA were examined using an Olympus BX-43 light microscope equipped with an Olympus DP12 digital camera and the image software Olympus DP-SOFT (Olympus, Tokyo, Japan). The entire smear was examined at high magnification (1000×). Representative preparations of sporozoites (accession nos. 49409NS-49425NS) were deposited at the Nature Research Centre, Vilnius, Lithuania.

2.3. DNA Extraction, PCR, and Sequencing

Total DNA was extracted from insect remnants using the ammonium acetate extraction method [27]. The extracted DNA was then dissolved in 20 µL of 1× TE solution. For genetic analysis, we used a nested PCR protocol which amplifies a fragment of 479 bp of the cytochrome b (*cytb*) gene of the *Haemoproteus* and *Plasmodium* parasites [28,29]. All samples were evaluated by electrophoresis using 2 µL of PCR product in a 2% agarose gel. One negative control (nuclease-free water) and one positive control (a sample with a single infection of *Plasmodium relictum cytb* lineage GRW4) were used in every run.

DNA fragments of all PCR-positive samples were sequenced in both directions with the corresponding primers using a Big Dye Terminator V3.1 Cycle Sequencing Kit and ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, Foster City, CA, USA). Electropherograms were analyzed using Geneious Prime 2022.2.1 for quality, identification of possible mixed infections (one peak for single infection, two or more peaks at the same position for mixed infections), and to create a contig sequence. Then, the contigs were analyzed and compared to other sequences using BLAST (Basic Local Alignment Search

Tool) in the MalAvi database (<http://130.235.244.92/Malavi/>, accessed on 1 July 2022) to determine parasite lineages. The sequences with at least one base-pair of difference from already deposited sequences were considered as new lineages [30]. All sequences were deposited in the GenBank (accession numbers OP546062–OP546095) and MalAvi databases.

To confirm some biting midges species or identify PCR-positive females from the *Culicoides obsoletus* group, we used the primers LCO1490 and HCO2198, which amplify a fragment of cytochrome *c* oxidase subunit I (*COI*) of the mitochondrial DNA of insects [31]. PCR products were sequenced from the 3' end with a Big Dye Terminator V3.1 Cycle Sequencing Kit and ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using BioEdit software, and obtained sequences were compared with other sequences using the BLAST on the GenBank. Identifications were considered for the sequences that presented similarity > 99%. Morphological identification was consistent with the PCR-based identification of the insects. These sequences were deposited in GenBank (accession numbers OP692758–OP692766).

2.4. Correlation between *Culicoides* Species and *Haemosporidian* Lineages

To understand the interactions between biting midges and their *Haemoproteus* parasites in Europe, studies published in the continent to date were used. To access these studies, we used the PubMed and Google Scholar databases; the search terms were: “*Culicoides* AND *Haemosporida*”, “*Culicoides* AND *Haemosporidian*”, “*Culicoides* AND *Haemoproteus*”, and “*Haemoproteus* AND vectors”. Only studies that used molecular methods and amplified a fragment of the *cytb* gene, available in the MalAvi database, were included in this analysis. Information on the vector species and parasite *cytb* gene lineages was used to create a database (Supplementary Table S1). For the studies that analyzed the samples using insect pools, each pool was considered as one sample.

Information for each *Culicoides* species containing the same *Haemoproteus cytb* lineage was summarized to obtain an interaction matrix, having as frequency of interaction consisting of the number of infected biting midges by a particular parasite lineage. A bipartite network and an adjacency matrix organized in modules of *Haemoproteus* lineages and *Culicoides* spp. were constructed in R 4.0.5 [32] using the bipartite package [33].

3. Results

3.1. Biting Midges and Parasite Diversity

In all, 580 parous *Culicoides* females belonging to 11 different species, all of which were reported in Lithuania, were collected and dissected (Table 1). The most abundant species were the *Culicoides obsoletus* group (31.9%), *Culicoides kibunensis* (19.7%), *Culicoides festivipennis* (15.3%), and *Culicoides punctatus* (11.9%). *Haemosporidian* parasite DNA was detected in 35 biting midges (6%), one being positive for *Plasmodium* DNA, and the remaining 34 biting midges positive for *Haemoproteus* DNA. Nine different genetic lineages of *Haemoproteus* were detected, including two new lineages, CULKIB02 and CULKIB03. Only one mixed infection was identified (Table 1).

3.2. Microscopic Analysis

Microscopic analysis of salivary gland preparations from the PCR-positive samples showed the presence of sporozoites in 17 samples (Figure 2), nine of them from *C. segnis* and eight from *C. kibunensis* (Table 1). Almost all *C. segnis* that were positive by PCR were also positive for sporozoites, except one. This is the first time that *C. segnis* has been confirmed as a competent vector for *H. fringillae* CCF3, *H. majoris* PHSIB1, *H. asymmetricus* TUPHI01, and *H. minutus* TURDUS2. This is also the first report of *C. kibunensis* being a competent vector for *H. belopolskyi* HIICT1.

3.3. Interaction Network between Biting Midge Species and *Haemoproteus* Lineages

The interaction network between *Culicoides*–*Haemoproteus* lineages (Figures 3 and 4) showed that, in nature, *C. kibunensis* is frequently associated with *H. asymmetricus* TUPHI01

and *H. minutus* TURDUS2. Additionally, *H. asymmetricus* TUPHI01 also presented a high number of interactions with *C. segnis* and *C. pictipennis*. Many *Haemoproteus* lineages were found interacting with only one biting midge species (e.g., *H. fringillae* CCF3 with *C. segnis*; and *H. concavocentralis* HAWF2 with *C. circumscriptus*). This might indicate a vector specialization by the *Haemoproteus* lineages encountered in the included studies. While *H. minutus* TURDUS2 was found interacting with ten different species of biting midges (Figure 4), showing low specificity in terms of vectors. On the other hand, most *Culicoides* species presented interactions with several lineages, suggesting that these insects are generalists in terms of parasite lineages. *Culicoides kibunensis* and *C. segnis* were the biting midge species with the highest number of interactions with different *Haemoproteus* parasites, with 13 each (Figures 3 and 4).

Table 1. Summary of collected *Culicoides* biting midges, with their respective *Haemoproteus* species and lineages.

<i>Culicoides</i> Species	n (Prevalence)	Haemosporidian <i>cytb</i> Lineage	Parasite Species (No. of PCR-Positive Insects)
<i>C. chiopterus</i>	2 (0)	-	-
<i>C. deltus</i>	1 (0)	-	-
<i>C. festivoipennis</i>	89 (4.5)	HIICT1 SYAT05	<i>Haemoproteus belopolskyi</i> (3) <i>Plasmodium vaughani</i> (1)
<i>C. impunctatus</i>	53 (0)	-	-
<i>C. kibunensis</i>	114 (14.9)	HIICT1 CULKIB02 CULKIB03 TUCHR01 TUPHI01 TURDUS2	<i>Haemoproteus belopolskyi</i> (4)¹ <i>Haemoproteus</i> sp. (1) <i>Haemoproteus</i> sp. (1) <i>Haemoproteus minutus</i> (1) <i>Haemoproteus asymmetricus</i> (4)² <i>Haemoproteus minutus</i> (6)³
<i>C. obsoletus</i> group *	185 (1.1)	HIICT1 WW2	<i>Haemoproteus belopolskyi</i> (2) <i>Haemoproteus majoris</i> (1)
<i>C. pallidicornis</i>	18 (5.3)	HIICT1	<i>Haemoproteus belopolskyi</i> (1)
<i>C. pictipennis</i>	2 (50)	TUPHI01	<i>Haemoproteus asymmetricus</i> (1)
<i>C. punctatus</i>	69 (0)	-	-
<i>C. reconditus</i>	1 (0)	-	-
<i>C. segnis</i>	42 (23.8)	CCF3 mix infection PHSIB1 TUPHI01 TURDUS2	<i>Haemoproteus fringillae</i> (2)¹ <i>Haemoproteus</i> spp. (1)¹ <i>Haemoproteus majoris</i> (1)¹ <i>Haemoproteus asymmetricus</i> (4)⁴ <i>Haemoproteus minutus</i> (2)⁴
<i>Culicoides</i> sp.	3 (0)	-	-

n = number of investigated insects. Bold indicates the *Haemoproteus* species from which sporozoites were found in salivary gland preparations. ¹ one sample positive for sporozoites; ² two samples positive for sporozoites; ³ five samples positive for sporozoites; ⁴ all samples were positive for sporozoites. (*) positive insects were molecularly confirmed to be *C. obsoletus*. (-) not evaluated.

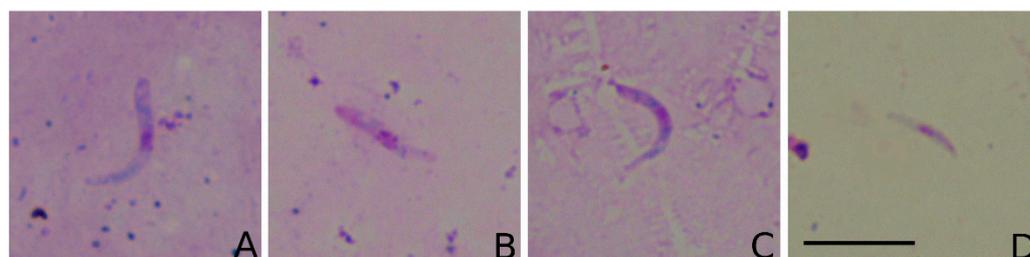


Figure 2. Sporozoites of *Haemoproteus asymmetricus* cytochrome *b* lineage TUPHI01 (A,C), *H. fringillae* CCF3 (B) in *Culicoides segnis*, and *H. minutus* TURDUS2 in *Culicoides kibunensis* (D) in salivary gland preparations. Scale bar: 10 μ m. Methanol-fixed and Giemsa-stained.

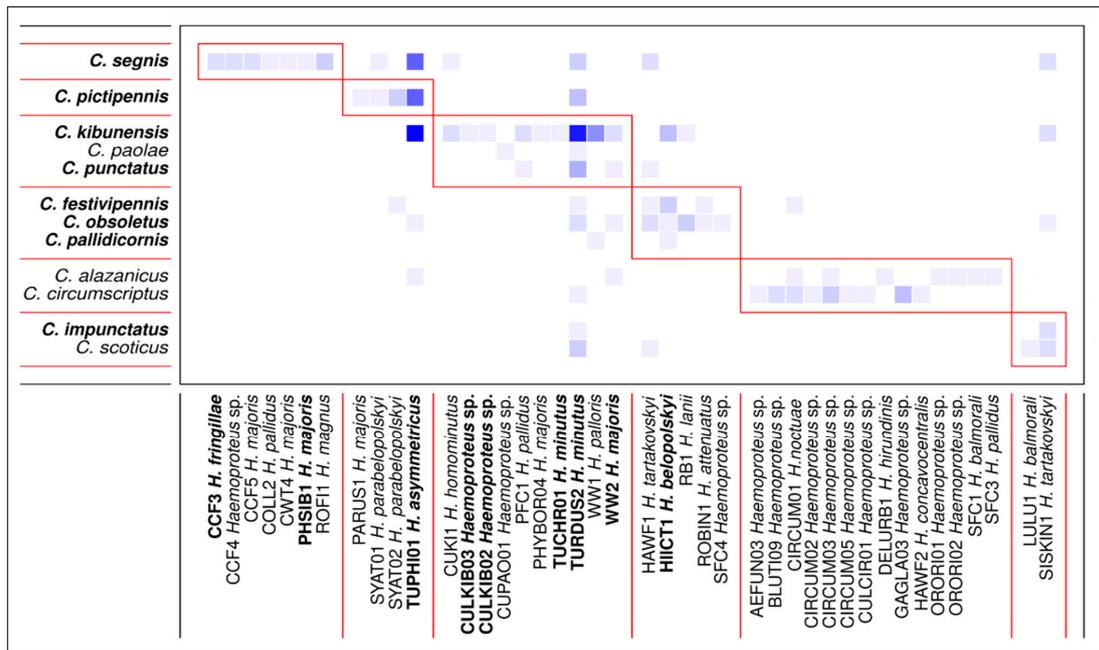


Figure 3. Modularity interaction network between *Culicoides* species (rows) and *Haemoproteus* lineages (columns). The blue squares represent the interactions between insects and parasites; the darker the square, the more often these interactions were registered. *Culicoides* species and *Haemoproteus* lineage investigated and found in this study are marked in bold. Red lines are separating different modules.

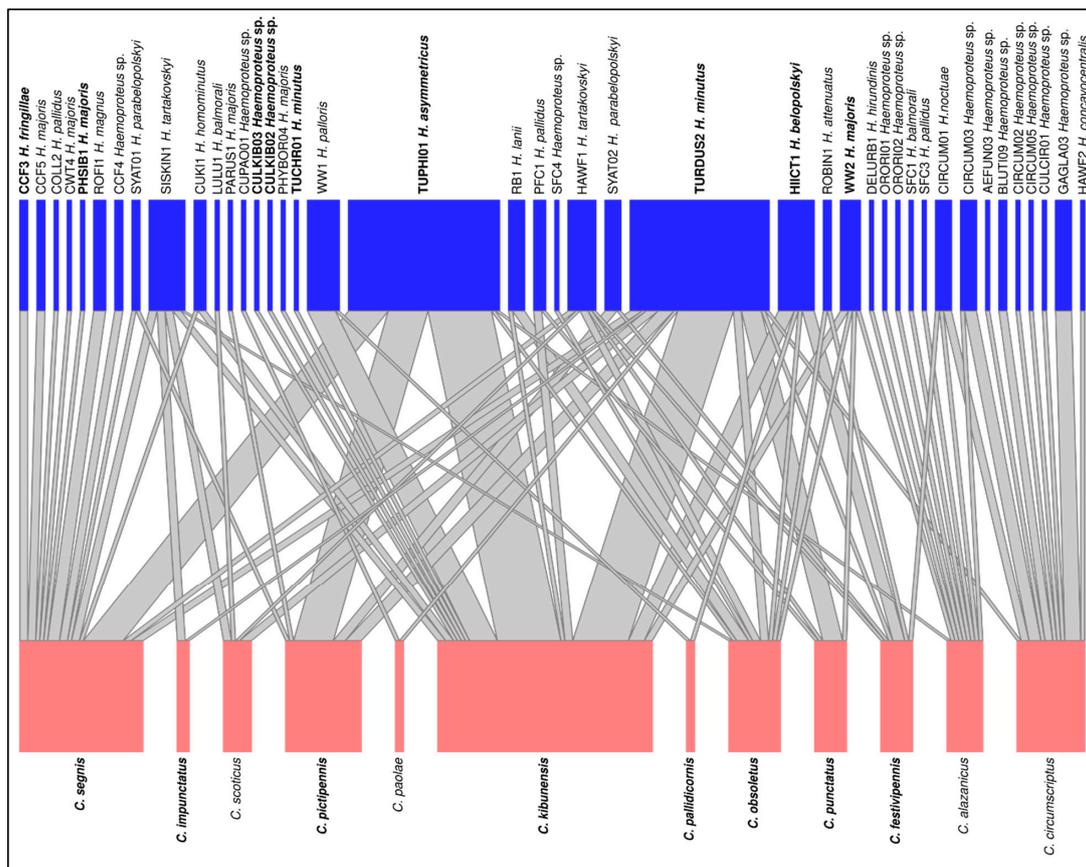


Figure 4. Bipartite interaction network between *Haemoproteus cytb* lineages (top, in blue boxes) and *Culicoides* species (bottom, in light red boxes). The width of the boxes is proportional to the frequency of the occurrence of a particular taxon. The width of connecting lines is proportional to the number of interactions.

of interactions observed between each *Culicoides* species and *Haemoproteus* lineage. *Culicoides* species and *Haemoproteus* lineages investigated and found in this study are marked in bold.

Modularity analysis detected six different modules (Figure 3), indicating that the transmission of *Haemoproteus* parasites in Europe presents a compartmentalized pattern, with no clear nestedness observed. In other words, different species or groups of species of *Culicoides* are more likely to transmit certain groups of *Haemoproteus* lineages.

4. Discussion

The key results of this study are the detection of sporozoites of *H. minutus* TURDUS2, *H. asymmetricus* TUPHI01, *H. majoris* PHSIB1, and *H. fringillae* CCF3 in salivary gland preparations from *C. segnis*, as well as sporozoites of *H. belopolnyi* HIICT1 in the salivary gland preparation of *C. kibunensis*, showing that they are competent vectors of these *Haemoproteus* parasites. *Culicoides kibunensis* and *C. segnis* seem to play an important role as vectors of *Haemoproteus* parasites, being reported interacting with many *Haemoproteus* species and lineages (Figures 3 and 4). On the other hand, some *Haemoproteus* parasites, e.g., *H. asymmetricus* TUPHI01, seem to be restricted to certain species of biting midges, such as *C. segnis*, *C. pictipennis* and *C. kibunensis*. Additionally, the number of interactions between *C. kibunensis* and *Haemoproteus* lineages described and commonly found in Turdidae birds (TURDUS2 and TUPHI01) was considerably high; this might indicate some feeding preference of *C. kibunensis* for Turdidae birds.

We found 11 different species of biting midges in our study (Table 1), all of them previously reported in Lithuania [34]. The most abundant species were biting midges from the *C. obsoletus* group and *C. kibunensis*, as previously reported in this country [3,5,6]. Other biting midge species seem to be rare, as is the case of *C. fagineus*, *C. reconditus*, *C. albicans*, *C. fascipennis*, *C. circumscriptus*, and *C. newsteadi* [3,5,6]. On the other hand, we reported the presence of *C. deltus*, which seems to be a rare biting midge species, and which was not recorded in the mentioned studies; even though this *Culicoides* species can be found all over Europe [25], it seems to be rare in the Eastern part of the continent [35]. Nevertheless, it is necessary to mention that, in some parts of Lithuania, *C. impunctatus* is the most abundant species [36]. This shows that even though several studies have been conducted in Lithuania, biting midge species diversity can vary from year to year, and between study sites, highlighting the importance of conducting more studies in different areas in the country to better understand the diversity of insects and their potential as vectors of *Haemoproteus* parasites.

The overall prevalence of *Haemoproteus* in *Culicoides* biting midges (5.9%) was similar to that noted in other studies conducted in Lithuania [3,5,6]. However, our results differed from other studies, with a prevalence higher than that in Bulgaria (approximately 2%) [8,9], as well as in Kaliningrad Oblast, Russia (1.7%) [7], but lower than that in Spain (13.4%) [10]. These differences should be carefully interpreted, since it depends on the study site, the density of insects, the diversity of the *Culicoides* species, their feeding preferences, the diversity of the bird species, the prevalence of *Haemoproteus* parasites in the bird populations, and the time of the year that the study was conducted.

Concerning the presence of *Haemoproteus* parasites in *C. obsoletus* group, the most abundant biting midge in the present study, we found a low prevalence of infections (only 1.1%). A study conducted in one of the areas that we sampled (Figure 1, area 1), did not report any infections in this biting midges species [6]. Interestingly, another study conducted in the same area (Figure 1, area 1) in 2016 reported a prevalence of approximately 6% [3], the same prevalence that was reported in the Curonian Spit, also in Lithuania [5]. This shows how dynamic infections can be in nature, and how much they can change between study sites and time of the year, increasing the complexity of understanding host-parasite-vector relationships. It is necessary to mention that, even though *Haemoproteus* DNA has been frequently found in *C. obsoletus*, showing that this biting midge species eventually feeds on birds, despite its mammalophilic behavior [17,19], *Haemoproteus* sporozoites have not been previously reported in this biting midge species.

The second-most abundant biting midge species, *C. kibunensis*, showed a high number of PCR-positive samples (14.9%), while a different positivity was previously reported in Lithuania, with 4.5% [3] and 45.5% [6] in Vilnius, and 7.8% in the Curonian Spit [5]. In the Czech Republic, the prevalence was of 51% of the insect pools analyzed [12], even though this insect species is distributed all over Europe [25]. In our study, *C. kibunensis* not only had a high number of PCR-positive females for *Haemoproteus*, but we also found sporozoites of *H. belopolskyi* HICT1 in the salivary gland preparations (Table 1, Figure 2), confirming that this parasite can be naturally transmitted by *C. kibunensis*. This biting midge species was already reported to be a competent vector for *H. pallidus* PFC1, *H. minutus* TURDUS2, and *H. asymmetricus* TUPHI01 [3,6]. *Culicoides kibunensis* has a certain flexibility in host selection, and even though it was reported to feed mainly on mammals, it also takes blood meals from birds [18].

Culicoides festiviipennis, another abundant biting midge species in our study, has been reported by several studies, including ours, to be positive for *Haemoproteus* DNA [3,5,8,12]. However, sporozoites were never found in salivary gland preparations. It might be that this biting midge species is a competent vector of *Haemoproteus* parasites, especially because of its ornithophilic feeding habits [19]. Furthermore, *Haemoproteus* infections have a relatively low prevalence in wild *Culicoides* [3,5–9], which means that a larger sampling might be necessary to prove the role of *C. festiviipennis* as a natural vector of *Haemoproteus* parasites.

Although *C. segnis* was not one of the most abundant biting midge species in the present study, it showed a high positivity in the PCR for *Haemoproteus* and a high positivity for sporozoites in the salivary gland preparations (Table 1). The prevalence was similar to the one previously reported in the Curonian Spit [5]. So far, *C. segnis* has been reported to be positive for *Haemoproteus* DNA only in Lithuania and the Czech Republic [5,6,12], while only recently, it has been proven to be a competent vector of *H. majoris* CCF5 and *H. tartakovskiyi* HAWF1 [5]. Our study adds three more species (four genetic lineages) of *Haemoproteus* to the list of species that *C. segnis* can transmit: *H. minutus* TURDUS2, *H. asymmetricus* TUPHI01, *H. fringillae* CCF3, and *H. majoris* PHSIB1.

For many years, a considerable number of experimental infections were performed with wild and laboratory-reared biting midges [4]. The most common experimental model is probably *C. impunctatus* [4], which was also collected and dissected in our study (Table 1). It has been shown to be a competent vector for at least 13 species of *Haemoproteus* parasites [4,16]. However, in this study all *C. impunctatus* were PCR-negative for *Haemoproteus* DNA, similar to the results reported in the literature, with no or a small prevalence of PCR-positive samples [6,7]. This means that, even though this biting midge species can transmit these parasites, the transmission probably does not occur in nature due to their mammalophilic habits. The fact that *Haemoproteus* DNA was found in those insects shows that they can sporadically feed on birds; however, to transmit *Haemoproteus* parasites, a second blood meal from a susceptible bird would be necessary (the sporozoite will take around seven days to develop after the first blood meal from an infected bird), and this might be very unlikely in nature. Additionally, these experiments were conducted with infected birds being held by hand, allowing biting midges to feed naturally [13,37]. The fact that *C. impunctatus* is highly attracted to humans [38,39] might induce some biased results, which would not reflect the natural transmission dynamics, even though proving that the biting midge can support parasite development, and it might not be a specialist at the vector level.

Another important characteristics of *C. impunctatus* that can directly affect *Haemoproteus* transmission is the fact that this biting midge species is considered to be bivoltine (having two generations per year) and autogenous (the first batch of eggs is produced without a blood meal) [38,40]. After laying their first batch of eggs, *C. impunctatus* females would become responsive to animal bait and to light, and if the biting midge can have a blood meal, then a second batch of eggs could be laid [41]. Due to vector biology, a third batch of eggs would be unlike, meaning that *C. impunctatus* females would not feed on a vertebrate host again and, even though they are infected by *Haemoproteus* parasites, they would not transmit it. However, there are only a few studies concerning this topic with

regards to *C. impunctatus* [38,41]. Thus, the lack of natural infections in this biting midge species in nature is probably the result of a combination of these features: mammalophilic habits, bivoltinism, and autogeny.

The interaction network analysis confirms this complex relationship between the parasites and their vectors, showing an intricate pattern (Figures 3 and 4). Our analysis showed that *C. kibunensis* plays an important role in the transmission of *Haemoproteus* parasites in Europe. Not only it was found interacting with several *Haemoproteus* lineages (13 in total), but it also represented the biting midge species with the highest diversity of *Haemoproteus* lineages in the present study. However, its main interactions were with *H. minutus* TURDUS2 and *H. asymmetricus* TUPHI01, both lineages were described in *Turdus merula* and *Turdus philomelos*, respectively; even though these lineages were reported in other bird species, their main hosts are Turdidae birds.

Culicoides segnis also presented a high number of interactions with *Haemoproteus* lineages (also 13), highlighting the interactions with *H. asymmetricus* TUPHI01. This might indicate that *C. segnis* also plays an important role in the transmission of certain *Haemoproteus* parasites in nature, even though sporozoites were only recently found in their salivary glands. It is likely that *C. segnis* has a broader range of feeding preference in terms of host species.

It is necessary to mention that *C. circumscriptus* also had a considerably high number of interactions with different *Haemoproteus* species (ten in total). This is also the only biting midge species that was found to be positive for *Haemoproteus* DNA lineages found in birds belonging to Strigidae (owls), Accipitridae (hawks), and Corvidae (crows and ravens) (Figures 3 and 4), according to the MalAvi database (accessed on September 2022 [30]). This might be explained by the fact that some species of *Culicoides* prefer to live in places at different heights [19,42]. Following this, *C. circumscriptus* was more frequently sampled at 20–26 m above the ground [43], where most species of the mentioned families live; while *C. kibunensis* was caught in higher numbers at ground level [42], where most Turdidae birds the majority of the day.

The fact that different biting midge species prefer different heights is one explanation for why we noticed those differences in the network interaction. This shows that the transmission of vector-borne diseases is complex and can be influenced by several different factors. First, feeding preference should be considered, since some biting midge species are mammalophilic and even though they sporadically feed on birds, they probably do not play an important role in *Haemoproteus* transmission. Second, the capacity of the parasite to influence the vector behavior, which was investigated for some avian *Plasmodium* parasites, which are closely related to *Haemoproteus*, and *Culex* mosquitoes [44–48]. However, such research has never been conducted using a *Culicoides*-*Haemoproteus* system. Third, and probably one of the main factors that should be taken into account when investigating vector-borne diseases, is the need to have an infected host, a competent vector, and a susceptible host in the same place and at the same time.

It is necessary to mention that all the parasites found in the present study infect birds (Table 1) [1]. This allowed us to access the information regarding all *Culicoides* that were PCR-positive for *Plasmodium*/*Haemoproteus* fed on birds, even though they did not have sporozoites in their salivary glands. This information is important, due to the lack of research on *Culicoides* feeding preferences.

In our study, one sample was positive for avian *Plasmodium* DNA, even though these parasites are not transmitted by *Culicoides* insects. This is not new, and has been frequently reported in the literature [3,7,8,10], indicating that the biting midge fed on an infected bird. However, since the parasite does not complete its development in biting midges, they cannot be considered vectors of *Plasmodium* parasites.

5. Conclusions

Culicoides segnis is a competent vector for *H. minutus* TURDUS2, *H. asymmetricus* TUPHI01, *H. majoris* PHSIB1, and *H. fringillae* CCF3; and *C. kibunensis* is a competent

vector for *H. belopolnyi* HIICT1. We are adding two new *Haemoproteus* lineages to the list of parasites transmitted in Europe and highlighting the important role that *C. segnis* and *C. kibunensis* play in the transmission of these parasites. The relationship between *Culicoides* biting midges and *Haemoproteus* parasites is complex and represents a challenge regarding understanding how these parasites are transmitted in nature. More studies focusing on insect biology and the identification of the natural vectors of *Haemoproteus* parasites (combining molecular tools and the investigation of sporozoites in salivary gland preparations) should be encouraged.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d14110957/s1>, Table S1: Studies in Europe investigating the presence of *Haemoproteus* parasites DNA in wild *Culicoides* biting midges used in the interaction network analysis.

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