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High susceptibility of the laboratory-reared biting midges *Culicoides nubeculosus* to *Haemoproteus* infections, with review on *Culicoides* species that transmit avian haemoproteids

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Research Article

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Abstract

Haemosporidian parasites belonging to *Haemoproteus* cause avian diseases, however, vectors remain unidentified for the majority of described species. We used the laboratory-reared biting midges *Culicoides nubeculosus* to determine if the sporogonic development of three widespread *Haemoproteus* parasites completes in this insect. The midges were reared and fed on one common blackbird, white wagtail and thrush nightingale naturally infected with *Haemoproteus minutus, Haemoproteus motacillae* and *Haemoproteus attenuatus,* respectively. The engorged females were dissected in order to follow their sporogonic development. Microscopic examination was used to identify sporogonic stages. Bayesian phylogeny based on partial cytochrome *b* gene was constructed in order to determine phylogenetic relationships among *Culicoides* species-transmitted haemoproteids. All three parasites completed sporogony. Phylogenetic analysis placed *Culicoides* species transmitted haemoproteids in one well-supported clade, proving that such analysis readily indicates groups of dipteran insects transmitting avian haemoproteids. Available data show that 11 species of *Culicoides* have been proved to support complete sporogony of 18 species of avian haemoproteids. The majority of *Culicoides* species can act as vectors for many *Haemoproteus* parasites, indicating the low specificity of these parasites to biting midges, whose are globally distributed. This calls for control of haemoproteid infections during geographical translocation of infected birds.

Introduction

Species of *Haemoproteus* (Haemosporida, Haemoproteidae) are important pathogens of birds due to their high prevalence in many bird populations, diseases and even mortality caused in some non-adapted avian host (Garvin *et al.*, 2003; Donovan *et al.*, 2008; Olias *et al.*, 2011; Pacheco *et al.*, 2011) Haemoproteosis belongs to a group of neglected parasitic diseases, yet it might result in severe infection in some birds (Cardona *et al.*, 2002; Cannell *et al.*, 2013; Valkiūnas, 2015). Much information is available about prevalence (Ishtiaq *et al.*, 2007; Latta and Ricklefs, 2010; Silva-Iturriza *et al.*, 2012; Neto *et al.*, 2015), genetic diversity (Szymanski and Lovette, 2005; Dimitrov *et al.*, 2010; Belo *et al.*, 2011; Ivanova *et al.*, 2015; Reeves *et al.*, 2015; Smith *et al.*, 2016) and phylogenetic relationships (Santiago-Alarcon *et al.*, 2010; Carlson *et al.*, 2013; Yoshimura *et al.*, 2014; Olsson-Pons *et al.*, 2015; Bensch *et al.*, 2016) of avian haemoproteids, but vectors, sporogonic development and patterns of transmission of these blood parasites have been insufficiently investigated.

Haemoproteus parasites are cosmopolitan in birds (Scheuerlein *et al.*, 2004; Ishtiaq *et al.*, 2007; Loiseau *et al.*, 2010; Smith *et al.*, 2015). They are transmitted by louse flies (Hippoboscidae) and biting midges (Ceratopogonidae). Species of subgenus *Parahaemoproteus* are transmitted by *Culicoides* biting midges (Garnham, 1966; Valkiūnas, 2005; Atkinson, 2008). Louse flies are responsible for transmission of a handful species parasitizing doves, pigeons and several species of marine birds (Atkinson, 2008; Levin *et al.*, 2012; Santiago-Alarcon *et al.*, 2012; Valkiūnas, 2015). Recent experimental studies showed that the wild-caught biting midge *Culicoides impunctatus* is susceptible to many *Parahaemoproteus* species (Žiegytė *et al.*, 2017), but information about other biting midges remain scanty, and it is still absent in all parts of the world except for North America (Fallis and Wood, 1957; Bennett and Fallis, 1960;Atkinson *et al.*, 1983; Garvin and Greiner, 2003) and Europe (Valkiūnas *et al.*, 2002; Žiegytė *et al.*, 2014;, 2016;, 2017; Bukauskaitė *et al.*, 2015).

It is important to note that several studies reported *Haemoproteus* parasite DNA in vectors using polymerase chain reaction (PCR)-based tools (Ishtiaq *et al.*, 2008; Martínez-de la Puente *et al.*, 2011; Njabo *et al.*, 2011). MtDNA sequences of both *Plasmodium* and *Haemoproteus* species were identified in wild-caught biting midges *Culicoides circumscriptus* (Ferraguti *et al.*, 2013). However, PCR-based tools detect parasite DNA regardless of the stage of development present in insects. For instance, DNA of ookinetes present in midgut or DNA from oocysts, that abort development in the insect, might be amplified. In other words, the presence of DNA of the parasite alone is not sufficient proof of an insect species ability to transmit the

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parasite (Valkiūnas *et al.*, 2013*a*). To identify the vectors of haemosporidian parasites, it is essential to determine the presence of sporozoites in the salivary glands. Experimental observations, microscopic examination and PCR-based diagnostic methods should be combined in order to obtain convincing data about vectors in wildlife populations.

Several studies used wild-caught biting midges for experimental exposure and investigation of the sporogonic development of *Haemoproteus* parasites. It was shown that several *Haemoproteus* species can be transmitted by numerous *Culicoides* species (Žiegytė *et al.*, 2017). For example, *Haemoproteus mansoni* (syn. *Haemoproteus meleagridis*) from wild turkeys completes sporogony and can be transmitted by *Culicoides edeny*, *Culicoides hinmani*, *Culicoides haematopodus*, *Culicoides arboricola* (Atkinson *et al.*, 1983) and *Culicoides knowltoni* (Atkinson *et al.*, 1988); *H. mansoni* (syn. *Haemoproteus canachites*) from grouse sporogonic development completes in *Culicoides sphagnumensis* (Fallis and Bennett, 1960). Methodology for infection of wild-caught biting midges with haemoproteids is developed (Valkiūnas, 2005) and was successfully applied in field studies (Bukauskaitė *et al.*, 2015; Žiegytė *et al.*, 2017). However, because of high mortality of wild-caught biting midges in laboratory conditions (Bukauskaitė *et al.*, 2016), it is difficult to use them in more delicate long-lasting experiments aimed at better understanding of host–parasite relationships. Experimental observations of laboratory-reared biting midges would be helpful in parasitology studies, particularly because the insect colonies are permanently available. That would provide opportunities for the all-year around the research of difficult designs, which is difficult or even hardly possible in case of many species of wild-caught biting midges.

Culicoides nubeculosus is widely distributed in the Palearctic (Mathieu *et al.*, 2012) and probably is a natural vector of avian haemoproteids. This species was colonized (Boorman, 1974) and is susceptible to several *Haemoproteus* species (Miltgen *et al.*, 1981; Bukauskaitė *et al.*, 2015; Žiegytė *et al.*, 2016). However, the range of *Haemoproteus* parasites, which can complete sporogony in this insect remains unclear. The aim of this study was to gain new knowledge about sporogony development of haemoproteids in the laboratory-reared biting midge *C. nubeculosus*. Here, we examined the development of three common *Parahaemoproteus* parasites in this experimentally exposed insect, i.e., *Haemoproteus minutus* (cytochrome *b* gene lineage hTURDUS2), *Haemoproteus motacillae* (hYWT2) and *Haemoproteus attenuatus* (hROBIN1). We also determined phylogenetic relationships among *Haemoproteus* species, which vectors have been identified, and reviewed the literature on *Culicoides* species-transmitted avian haemoproteids.

Materials and methods

Study site, selection of experimental birds, collection of blood samples and microscopic examination

Experiments were carried out at the Ventės ragas Ornithological station, Lithuania (https://vros.lt) in May of 2015 and 2017. Birds were caught with mist nets, 'Zigzag' traps and a big 'Rybachy' type trap. The blood was collected from the brachial vein using heparinized microcapillaries, stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0). The samples were kept at ambient temperature in the field and were preserved at −20 °C at the laboratory. A small amount of freshly obtained blood was used to make three blood smears, which were rapidly dried using a battery-operated fan, fixed with absolute methanol and stained with 10% Giemsa solution, as described in Valkiūnas (2005).

Preparations were examined using Olympus BX-43 light microscope equipped with Olympus SZX2-FOF digital camera and imaging software QCapture Pro 6.0, Image Pro plius (Tokyo, Japan). Blood smears were examined for 15–20 min at low magnification (×400), and when approximately 100 fields were studied at high magnification (×1000). The intensity of gametocytaemia was determined as a percentage by actual counting of the number of mature gametocytes (Fig. 1A–F) per 1000 red blood cells. All vectors preparations were examined at high (×1000) magnification. Statistical analyses were carried out using the 'R studio' version 3.4.3. Student's *t*-test for independent samples was used to determine statistical significance between the mean linear parameters of parasites. A P value of 0.05 or less was considered significant. Representative preparations for vector stages (49011 NS – 49018 NS) were deposited in Nature Research Centre, Vilnius, Lithuania.

Experimental design

Birds naturally infected with single *Haemoproteus* infections were used as donors of gametocytes to expose biting midges. One common blackbird *Turdus merula*, one white wagtail *Motacilla alba* and one thrush nightingale *Luscinia luscinia* infected with *H. minutus* (cytochrome *b* gene lineage hTURDUS2), *H. motacillae* (hYWT2) and *H. attenuatus* (hROBIN1), respectively were used to infect biting midges. All experimental birds survived and were released approximately 1.5 h after their capture at the study site.

Culicoides nubeculosus biting midges were reared in the laboratory according to Boorman (1974). Briefly, they were kept in small cardboard boxes covered with fine mesh bolting silk. Each box contained approximately 50–70 individuals of biting midges. A box with unfed insects was gently pressed to the feather-free area on pectoral muscles of infected birds. The midges willingly took blood meal through the bolting silk, and the majority of them were fully engorged approximately after 30–40 min. Then, the experimental insects were released in a cage made of bolting silk ($12 \times 12 \times 12$ cm^3) and males and non-fed females were removed. The remaining engorged biting midges were kept in a room with controlled temperature (22 °C), humidity (75 ± 5%) and light-dark photoperiod (17:7 h). Cotton pads moistened with 10% solution of sugar were placed on the top of each cage in order to feed insects daily.

Dissection of biting midges and making preparations of parasites

Experimentally infected biting midges were dissected and preparations of ookinetes, oocysts and sporozoites were prepared. Before dissections, the insects were anesthetized by placing them in a tube covered with cotton-wool pads moistened with 96% ethanol. Preparations of ookinetes were made by extraction of the midgut, which was gently crushed on the slide. One thin film was prepared from each insect, and the preparation was fixed with methanol and stained the same way as blood films. In order to visualize oocyst, midguts were gently isolated on objective slides and a drop of 2% mercurochrome solution was placed on each of them. Then, the midguts were covered with cover-slips and oocysts were visualized in these temporary preparations. Permanent oocyst preparations were made according to Valkiūnas (2005). Briefly, midguts were fixed in 10% normal formalin solution for 24 h. Then, formalin was replaced by placing the preparations in 70% ethanol for 6 h. The preparations were washed with distilled water, stained with Ehrlich's hematoxylin for 10 minutes, steeped in water with a pinch of sodium bicarbonate and differentiated with acid-ethanol for 5 min and steeped again in water with sodium bicarbonate. Then, the preparations were dehydrated with 70% and then with 96% ethanol. A drop of clove oil and xylene was used to clear the preparation.

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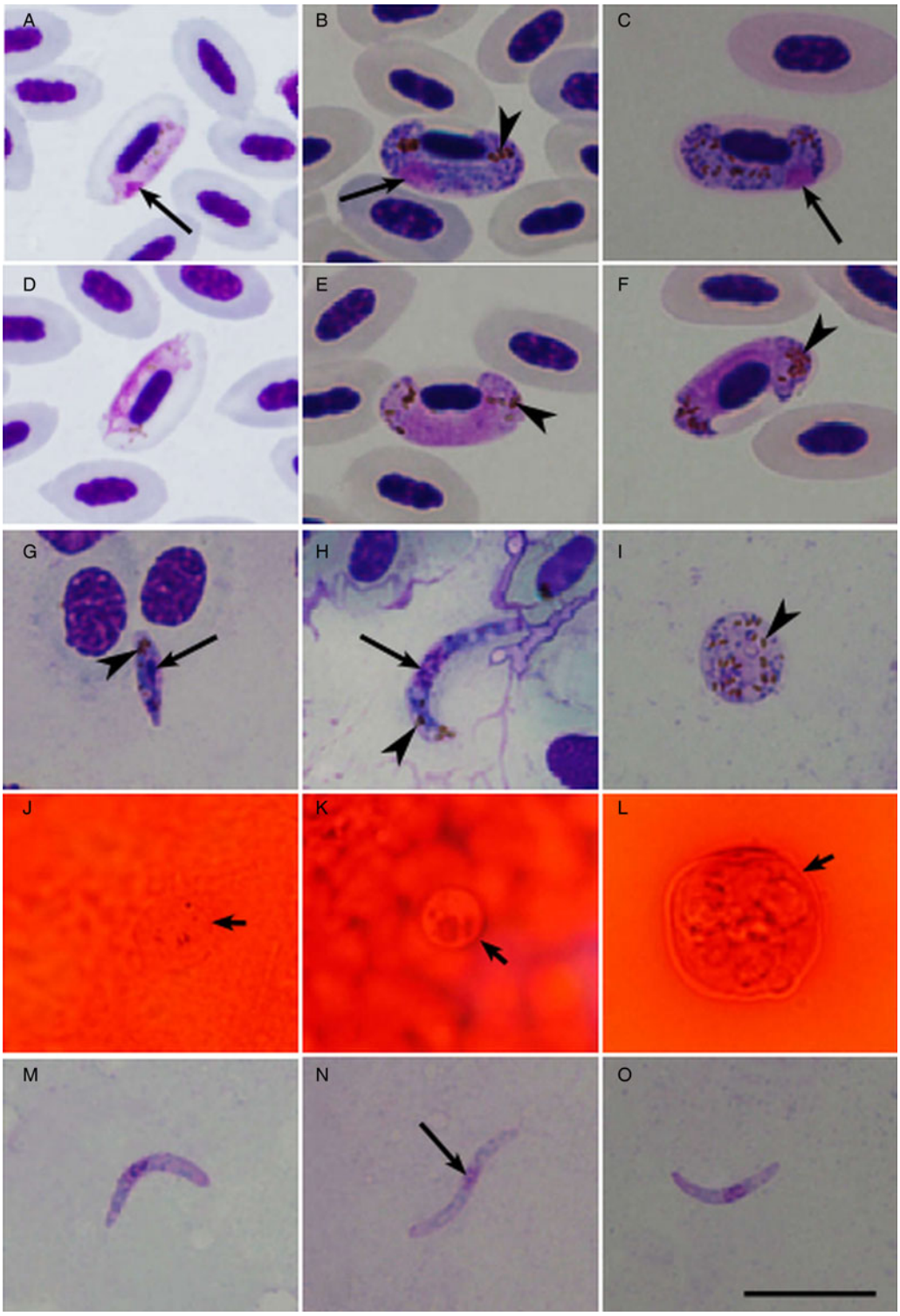

Fig. 1. Gametocytes (A–F) and sporogonic stages (G–O) of *Haemoproteus minutus* (lineage hTURDUS2) (A, D, G, J, M), *Haemoproteus motacillae* (hYWT2) (B, E, H, K, N) and *Haemoproteus attenuatus* (hROBIN01) (C, F, I, L, O) in *Culicoides nubeculosus*. Mature macrogametocytes (A–C) and microgametocytes (D–F) in the blood of common blackbird *Turdus merula* (A, D), white wagtail *Motacilla alba* (B, E) and thrush nightingale *Luscinia luscinia* (C, F). Ookinetes (G, H), zygote (I), oocysts (J–L) and sporozoites (M–O) in *Culicoides nubeculosus* preparations. Methanol-fixed and Giemsa-stained thin films (A–I, M–O). Formalin-fixed whole mounts stained with hematoxilin (J–L). Long simple arrows – nuclei of parasites, simple arrowheads – pigment granules, short arrow – oocysts. Scale bar = 10 μm.

Finally, a drop of Canada balsam was placed on midguts, which were covered with cover-slips and air-dried for several days.

Preparations of sporozoites were made by extracting the salivary glands from biting midges and gently crashing them to prepare small thin smears, which were fixed with absolute methanol and stained with 4% of Giemsa solution for 1 hour.

After each insect dissection, residual parts of their bodies were fixed in 96% ethanol and used for PCR-based analysis in order to confirm the presence of corresponding parasite lineages in vectors. Dissected needles were disinfected in the fire to prevent contamination after each dissection.

Polymerase chain reaction, sequencing and phylogenetic analysis

Total DNA was extracted from all samples using the standard ammonium acetate extraction method (Richardson *et al.*, 2001). Nested PCR protocol was used to amplify *cytb* gene fragment

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Table 1. Morphometry of sporozoites of three *Haemoproteus* species in the biting midge *Culicoides nubeculosus*

	Measurements[a]		
Feature	*H. minutus* (*N* = 21)	*H. motacillae* (*N* = 18)	*H. attenuatus* (*N* = 21)
Length	9.9–12.8 (11.8 ± 0.7)	9.0–12.4 (10.4 ± 1.0)	7.6–10.9 (9.5 ± 0.9)
Width	1.2–1.6 (1.4 ± 0.1)	1.1–1.7 (1.4 ± 0.2)	0.8–1.5 (1.2 ± 0.2)
Area	10.55–17.0 (13.1 ± 1.5)	8.7–16.4 (13.0 ± 2.1)	6.4–13.7 (9.7 ± 1.8)

[a]All measurements are given in micrometres. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

(Bensch *et al.*, 2000; Hellgren *et al.*, 2004). The primers HaemNFI and HaemNR3 were used to amplify fragments of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* parasites. The primers HaemF and HaemR2 were applied for the second PCR, which amplifies DNA of *Haemoproteus* and *Plasmodium* parasites. The success of the performed PCR was evaluated by running 1.5 µL of PCR product on a 2% agarose gel. One negative control (nuclease-free water) and one positive control (a *Haemoproteus* sp. infected sample, which was positive by microscopic examination) were used every 7 samples to control for possible false amplifications.

Fragments of DNA from the PCR positive amplifications were sequenced. Big Dye Terminator V3.1 Cycle Sequencing Kit and ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, Foster City, California) were used for sequencing. Sequences were edited and aligned using BioEdit software (Hall, 1999). The genetic analyser 'Basic Local Alignment Search Tool' (National Centre of Biotechnology Information website: http://www.ncbi.nlm.nih.gov/BLAST) was used to compare detected sequences to those deposited in the GeneBank.

To determine the phylogenetic relationship among *Haemoproteus* parasites, which vectors have been identified, we constructed a phylogenetic tree using 33 sequences of the mitochondrial *cytb* gene. Three sequences of *Plasmodium* spp. were also used to increase the resolution of the phylogenetic reconstruction. *Leucocytozoon* sp. was used as an outgroup. The tree was developed using a Bayesian algorithm (MrBayes version 3.1; Ronquist and Heulsenbeck, 2003). Best fit model of evolution (GTR+I+G) was selected by software Modeltest 3.7 (Posada and Crandall, 1998). The analysis was run for a total of 10 million generations with a sample frequency of every 100th generation. Before the construction of the consensus tree, 25% of the initial trees was discarded as 'burn in' period. The tree was visualized using the software FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The absence of double-base calling in sequence electropherograms was used as an indication of single infections (Pèrez-Tris and Bensch, 2005).

Results

Both microscopic examination (Fig. 1A–F) and PCR-based testing showed the presence of single *Haemoproteus* infections in donor birds. *Haemoproteus minutus* (*cytb* lineage hTURDUS2), *H. motacillae* (hYWT2) and *H. attenuatus* (hROBIN1) were present in donor common blackbird, white wagtail and trush nightingale, respectively. PCR and sequencing confirmed the presence of corresponding parasites lineages in experimentally infected insects.

Sporogony of Haemoproteus parasites in Culicoides nubeculosus

Sporogony of all three parasite species occurred and completed in experimentally infected biting midges *C. nubeculosus* (Fig. 1G–O). Sporozoites of *H. minutus*, *H. motacillae* and *H. attenuatus* developed and were visualized in salivary glands preparation (Fig. 1M–O).

Mature ookinetes of *H. minutus* (Fig. 1G) were reported in midguts 1–2 h post infection (hpi), while the ookinetes of *H. motacillae* (Fig. 1H) were detected 12 hpi, indicating the markedly different rate of development in these parasites. Only zygotes of *H. attenuatus* were seen between 3 and 6 hpi (Fig. 1I). Ookinetes of this parasite possibly develop later, but the preparations were not made on later hours.

Oocysts of all three species were seen in exposed biting midges. Oocyst of *H. minutus* (Fig. 1J) were seen 3–5 days post infection (dpi) and oocysts of *H. motacillae* (Fig. 1K) and *H. attenuatus* (Fig. 1L) were reported 3–4 dpi and 5–7 dpi, respectively.

Sporozoites of *H. minutus* were reported in salivary gland preparations 7 dpi (Fig. 1M). Sporozoites of *H. motacillae* (Fig. 1N) and *H. attenuatus* (Fig. 1O) were seen 8–9 dpi and 6–9 dpi, respectively. Measurements of sporozoites are given in Table 1. There was a significant difference in sporozoite length between all parasite species. Sporozoites of *H. minutus* were longer than those of *H. motacillae* ($p < 0.001$) and *H. attenuatus* ($p < 0.001$) and sporozoites of *H. motacillae* were longer than in *H. attenuatus* ($p = 0.005$). There was no difference discernible between *H. minutus* and *H. motacillae* in sporozoite width ($p = 0.96$) and area ($p = 0.86$). However, sporozoites of *H. attenuatus* were significantly thinner and smaller in area than sporozoites of *H. motacillae* ($p = 0.008$, $p < 0.001$, respectively) and *H. minutus* ($p = 0.002$, $p < 0.001$, respectively).

Phylogenetic analysis

Three species of *Haemoproteus* used in this study appeared in one well-supported clade with other *Culicoides* species-transmitted parasites (Fig. 2, clade A). These parasites belong to subgenus *Parahaemoproteus*. Species of subgenus *Haemoproteus,* which are transmitted by louse flies appeared in a separate well-supported clade (Fig. 2, clade B).

Discussion

The key result of this study is that three species of *Haemoproteus* completed sporogonic development in laboratory-reared biting midge *C. nubeculosus*. Sporozoites of *H. minutus, H. motacillae* and *H attenuatus* were seen in salivary gland preparations (Fig. 1M–O), indicating that this biting midge likely is the natural vectors of these parasites. All these haemoproteids are parasites of passeriform birds. Žiegytė *et al.* (2016) also reported complete sporogony of *Haemoproteus (Parahaemoproteus) tartakovskyi,* the parasite of passeriform birds in *C. nubeculosus*. However, there are also experimental data showing that this biting midge is susceptible and support the complete sporogonic development of *Haemoproteus* species parasitizing birds belonging to other orders, i.e. *Haemoproteus (Parahaemoproteus) handai*, the parasite of parrots belonging to Psittaciformes (Miltgen *et al.*, 1981) and *Haemoproteus (Parahaemoproteus) noctuae* and *Haemoproteus*

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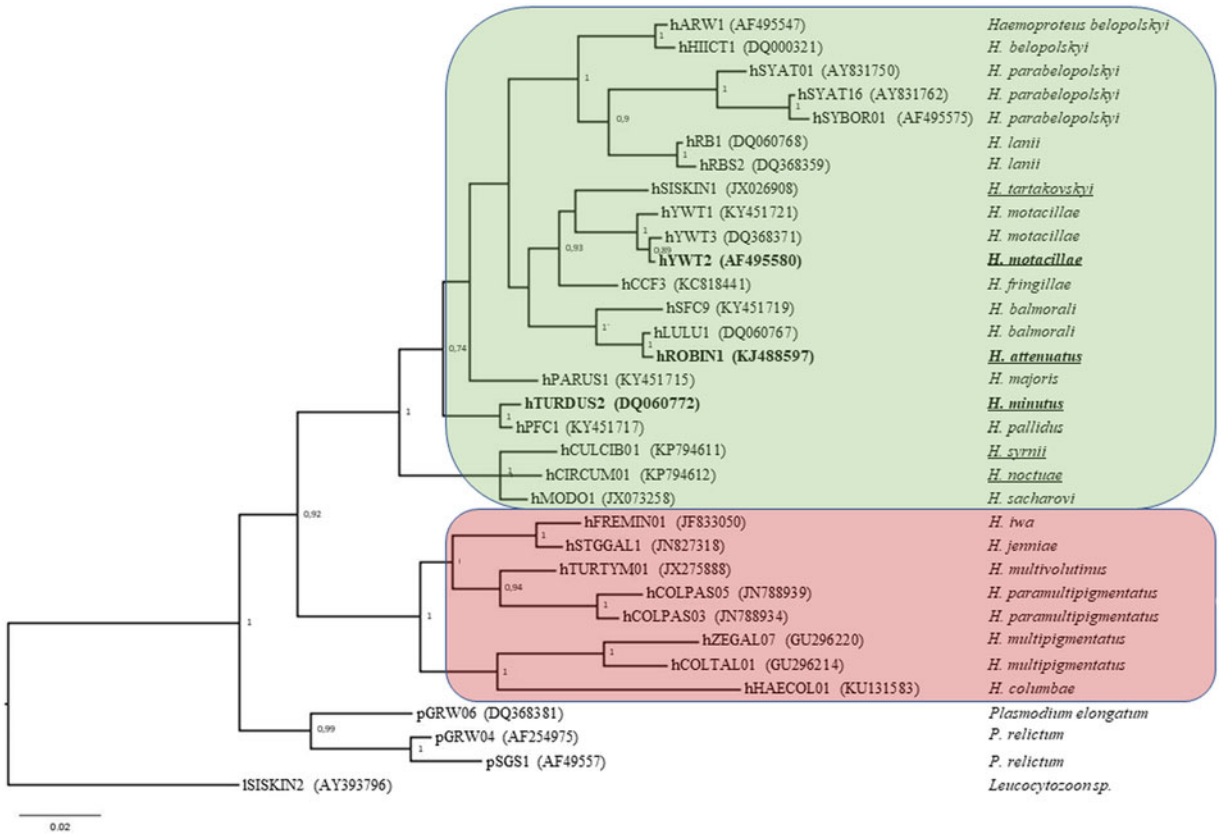

Fig. 2. Bayesian phylogeny of mitochondrial cytochrome *b* lineages (479 bp) of avian *Haemoproteus* parasites, which are transmitted by biting midges (clade A, green box) and louse flies (clade B, red box). The tree was rooted with *Leucocytozoon* sp. (lineage SISKIN2) and it is drawn to scale based on inferred substitutions per site. Nodal support values indicate Bayesian posterior probabilities. Codes of the lineages are given according to MalAvi database (Bensch *et al*., 2009). GenBank accession numbers of sequences are provided in the parenthesis. Data about parasites used in this study are given in bold font. Parasite species completing sporogony in *Culicoides nubeculosus* were underlined.

(Parahaemoproteus) syrnii, the parasites of owls belonging to Strigiformes (Bukauskaitė *et al*., 2015). In all, the available data show that seven species of avian haemoproteids parasitizing birds of three orders can use *C. nubeculosus* as final host and the vector (Fig. 2). According to limited available data, *C. nubeculosus* is not specialized in the blood meal. This biting midge willingly takes blood from various mammals and birds (Jennings and Mellor, 1988). Recent experimental studies, which applied experimental exposure of sheep (Pages *et al*., 2014), canaries (Svobodova *et al*., 2017), owls (Bukauskaitė *et al*., 2015) and various species of wild passeriform birds (Žiegytė *et al*., 2016) support this conclusion. Further field studies using molecular markers are needed for better understanding the feeding preferences of *C. nubeculosus* in different ecosystems. This insect is highly susceptible to various *Haemoproteus* infections and likely participate in the natural transmission of haemoproteosis. Due to broad distribution in Eurasia (Mathieu *et al*., 2012), *C. nubeculosus* should be considered as important haemoproteid vector, which is worth more attention in parasitology research.

Sporozoites of *H. minutus*, *H. motacillae* and *H. attenuatus* were reported in salivary glands on 7, 8–9 and 6–9 dpi, respectively (this study), while the sporozoites of *H. noctuae*, *H. syrnii* and *H. tartakovskyi* were seen between 7 and 11 dpi (Bukauskaitė *et al*., 2015; Žiegytė *et al*., 2016). Sporozoites of *H. handai* were first reported in salivary glands 5 dpi (Miltgen *et al*., 1981). In experimental research at a temperature of approximately 20–22 C, we advise to access sporozoites in exposed insects between 7 and 9 dpi when they are numerous in salivary glands.

Bukauskaitė *et al*. (2015) showed that morphologically identical sporozoites of *H. noctuae* developed in *C. nubeculosus* and *C. impunctatus*, while sporozoites of different parasites species (*H. noctuae* and *H. syrnii*) were morphologically different and readily distinguishable during development in *C. nubeculosus* biting midge. Sporozoites of *H. syrnii* were significantly smaller both in length and in area than those of *H. noctuae*. This shows that the size of sporozoites might be used as a taxonomic character in distinguishing avian haemoproteids in vectors. In this study, the sporozoites of *H. attenuatus* were significantly shorter than those of *H. minutus* and *H. motacillae* (Fig. 1M–O, Table 1). Ookinetes of different *Haemoproteus* species also markedly vary in size and shape (compare Fig. 1G and H) and were proved to be readily distinguishable in many avian haemoproteid species during development both *in vivo* and *in vitro* (Valkiūnas, 2005; Valkiūnas *et al*., 2013*b*; Žiegytė *et al*., 2017).

Available data show that *C. nubeculosus* is susceptible to seven *Haemoproteus (Parahaemoproteus)* species (Table 2). *Culicoides impunctatus* is also the highly susceptible species of biting midges, which support complete sporogony of 11 *Parahaemoproteus* parasites (Table 2). *Culicoides impunctatus* is widespread in the Palearctic and is abundant in Europe (Glukhova and Valkiūnas, 1993; Blackwell *et al*., 1999; Patakakis *et al*., 2009). Experimental studies show that infective sporozoites of haemoproteids develop in this biting midge (Valkiūnas *et al*., 2002), which certainly is an effective natural vector. Wild-caught *C. impunctatus* insects were used in many experimental studies (Žiegytė *et al*., 2014; Bukauskaitė *et al*., 2015; Žiegytė *et al*., 2017). However, experimental research with wild-caught *C. impunctatus* insects is limited due to a short-period (several weeks in spring-summer time) when they are very abundant and are easy to sample in large numbers for experimental infections at many study sites in Europe

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Table 2. Species of *Culicoides* biting midges that support the complete sporogonic development of avian *Haemoproteus* parasites of subgenus *Parahaemoproteus*

Vector	*Haemoproteus* species	Reference
Culicoides arboricola	*Haemoproteus danilewskii*	Garvin and Greiner (2003)
	Haemoproteus mansoni[a]	Atkinson *et al.* (1983)
Culicoides crepuscularis	*Haemoproteus danilewskii*	Bennett and Fallis (1960)
	Haemoproteus fringillae	Fallis and Bennett (1961)
Culicoides downesi	*Haemoproteus nettionis*	Fallis and Wood (1957)
	Hemoproteus danilewskii	Fallis and Bennett (1961)
Culicoides Edeni	*Haemoproteus danilewskii*	Garvin and Greiner (2003)
	Haemoproteus mansoni[a]	Atkinson *et al.* (1983)
Culicoides haematopotus	*Haemoproteus mansoni*[a]	Atkinson *et al.* (1988)
Culicoides hinmani	*Haemoproteus mansoni*[a]	Atkinson *et al.* (1983)
Culicoides impunctatus	*Haemoproteus balmorali*	Valkiūnas *et al.* (2002)/Žiegytė *et al.* (2017)
	Haemoproteus belopolskyi	Valkiūnas and Iezhova (2004*a*)/Žiegytė *et al.* (2014)
	Haemoproteus dolniki	Valkiūnas *et al.* (2002)
	Haemoproteus fringillae	Valkiūnas (1997)
	Haemoproteus lanii	Valkiūnas and Iezhova (2004*b*)
	Haemoproteus majoris	Žiegytė *et al.* (2017)
	Haemoproteus minutus	Žiegytė *et al.* (2014)
	Haemoproteus motacillae	Žiegytė *et al.* (2017)
	Haemoproteus noctuae	Bukauskaitė *et al.* (2015)
	Haemoproteus pallidus	Žiegytė *et al.* (2017)
	Haemoproteus parabelopolskyi[b]	Valkiūnas and Iezhova (2004*b*)
	Haemoproteus tartakovskyi	Valkiūnas *et al.* (2002)
Culicoides knowltoni	*Haemoproteus danilewskii*	Garvin and Greiner (2003)
	Haemoproteus mansoni[a]	Atkinson *et al.* (1988)
Culicoides nubeculosus	*Haemoproteus attenuatus*	This study
	Haemoproteus handai	Miltgen *et al.* (1981)
	Haemoproteus minutus	This study
	Haemoproteus motacillae	This study
	Haemoproteus noctuae	Bukauskaitė *et al.* (2015)
	Haemoproteus syrnii	Bukauskaitė *et al.* (2015)
	Haemoproteus tartakovskyi	Žiegytė *et al.* (2016)
Culicoides sphagnumensis	*Haemoproteus danilewskii*	Fallis and Bennett (1961)
	Haemoproteus fringillae	Fallis and Bennett (1961)
	Haemoproteus mansoni[c]	Fallis and Bennett (1960)
	Haemoproteus velans	Khan and Fallis (1971)
Culicoides stilobezziodes	*Haemoproteus danilewskii*	Bennett and Fallis (1960)
	Haemoproteus fringillae	Fallis and Bennett (1961)
	Haemoproteus velans	Khan and Fallis (1971)

[a]Originally (Atkinson *et al.* 1983, 1988), this parasite was attributed to *Haemoproteus meleagridis*.
[b]Originally (Valkiūnas *et al.* 2007), this parasite was attributed to *Haemoproteus belopolskyi*.
[c]Originally (Fallis and Bennett 1960), this parasite was attributed to *Haemoproteus canachites*.

(Liutkevičius, 2000). This complicates the use of wild-caught insects in experimental research in wildlife. Additionally, mortality of wild-caught *C. impunctatus* insects is high in captivity (Valkiūnas and Iezhova, 2004*b*; Bukauskaitė *et al.*, 2016). Meanwhile, *C. nubeculosus* is easy to rear at the laboratory conditions and survive well (Boorman, 1974). We recommend using this insect in experimental *Haemoproteus* parasite studies, which can be designed and carried out all the year round. This opens new opportunities for delicate experimental observations on various aspects of haemoproteid parasite biology.

As far, 11 species of biting midges have been proved to support complete sporogony of avian *Haemoproteus* parasites (Table 2). The majority of tested *Culicoides* species supported sporogony of several (up to 11) species of avian *Haemoproteus*. This provides an opportunity to conclude about the low specificity of *Culicoides* biting midges to *Haemoproteus* parasites, each species of the latter can use different biting midges for transmission. That contributes to better understanding of the cosmopolitan distribution of many avian *Haemoproteus* species (Valkiūnas, 2005), which likely can assess susceptible *Culicoides* vectors in various ecosystems.

Phylogenetic analyses placed all species transmitted by *C. nubeculosus* and other *Culicoides* spp.-transmitted haemoproteids in a separate well-supported clade (Fig. 2, clade A), which contains parasites of subgenus *Parahaemoproteus*. Parasites of subgenus *Haemoproteus* appeared in the sister clade (Fig. 2, clade B), and they are transmitted by louse flies (Valkiūnas, 2005, 2015;

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Atkinson, 2008; Levin *et al.*, 2012; Santiago-Alarcon *et al.*, 2012). This result supports and strengthens conclusions of former studies, which suggest the use the phylogenies based on partial *cytb* genes for estimation groups of most possible vectors (biting midges or louse flies), which are involved in haemoproteid transmission. Such preliminary estimation is useful due to difficulties in experimental research with parasites of wild birds, particularly protected species and during research in remote areas. Phylogenetic relationships provide clear links to most possible vector groups of *Haemoproteus* parasites and are recommended as a useful easy tool before planning expensive and difficult to design experimental vector research. However, the experimental observations and vector dissection remain essential for a final demonstration in which insect species infective sporozoites develop and sporogony is completed (Bukauskaitė *et al.*, 2015; Žiegytė *et al.*, 2017).

It is interesting to note that phylogenies based both on partial *cytb* gene and complete mtDNA of haemosporidian parasites show that major parasite clades were associated with certain dipteran vector groups. Mainly, haemosporidians transmitted by species of Hippoboscidae, Ceratopogonidae, Simuliidae and Culicidae appeared in separate well-supported clades (Martinsen *et al.*, 2008; Bukauskaitė *et al.*, 2015; Žiegytė *et al.*, 2017; Pacheco *et al.*, 2018). Relatively strict linkage of haemosporidian parasites of particular clades to specific insect families remain insufficiently understood, and there is no convincing explanation of this observation. It might be an indication that development of vectors may be essential in the selection of the drive of evolution in mtDNA genes in the haemosporidian parasite. These protists use both glycolysis and oxidative phosphorylation energy metabolism during their life cycle. However, the glycolysis predominates during haemosporidian development in the vertebrate host, but the parasites switch mainly to oxidative phosphorylation in the insect vectors, in which glucose is insufficiently available for adenosine triphosphate synthesis (Hino *et al.*, 2012). Thus, the mitochondrial genes are crucial for survival haemosporidians in vectors, but not so important during development in vertebrates, in which glycolysis predominates (Hall *et al.*, 2005; Jacot *et al.*, 2016; Pacheco *et al.*, 2018). Because dipteran insects belong to genetically different groups and are markedly different environments for parasites, this should be reflected in phylogenies based on mitochondrial DNA genes. In other words, the phylogenies based mtDNA may reflect well the parasite–vector evolutionary relationships, but not so well the modes of evolution of entire 'vertebrate host – parasite – vector' system. That might explain the contradictions in haemosporidian phylogenies based on different genes (Bensch *et al.*, 2016). Analysis of phylogenies based on complete haemosporidian genomes is needed to answer this question. However, such analysis is currently premature due to still limited taxon sampling in wildlife haemosporidian parasites on the genomic level.

It is worth noting that Miltgen *et al.* (1981) experimentally exposed *C. nubeculosus* to *H. handai* (syn. *Haemoproteus desseri*) infection and demonstrated the development of numerous sporozoites in salivary glands, indicating the high vectorial ability of this insect. A naturally infected Blossom-headed parakeet *Psittacula roseata* imported from Thailand to France was used as a donor of gametocytes of this parasite to expose the biting midges. *Haemoproteus handai* is a specific parrot infection, which normally does not occur in European birds (Valkiūnas, 2005). Due to the high susceptibility of *C. nubeculosus* not only to European haemoproteids (Table 2) but also the parasites of tropical exotic birds, this blood-sucking insect might contribute to the distribution of invasive haemoproteosis in regions where such infections are absent. *Haemoproteus* parasites are relatively specific to vertebrate hosts, and the same pathogen usually does not complete a life cycle in birds belonging to different orders (Valkiūnas, 2005). This is a natural obstacle for the establishment of the new nidus of *Haemoproteus* infections in wildlife. However, *Haemoproteus* infections might proceed partially in unusual avian hosts and produce tissue stages, which then abort development, but cause severe pathology and even mortality in birds (Donovan *et al.*, 2008; Olias *et al.*, 2011). Such infections are difficult to diagnose, and treatment of them remains non-developed. We advise the responsible veterinary agencies to the attentive control of the import of birds for haemoproteid infections because local biting midges of *Culicoides* are highly susceptible to many *Haemoproteus* species and might transmit new diseases to wildlife and poultry (Opitz *et al.*, 1982; Valkiūnas and Iezhova, 2017).

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