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A survey of biting midges of the genus *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) in NE Bulgaria, with respect to transmission of avian haemosporidians

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Abstract

This study presents data from a molecular survey of the species of the genus *Culicoides* from the region of Kalimok Field Station (NE Bulgaria) and haemosporidian parasites occurring in them in order to investigate the host-parasite specificity of haemosporidians to their dipteran vectors. The identification of *Culicoides* spp. was carried out by morphological and molecular-genetic methods. We collected and analysed 230 individuals of the genus *Culicoides*. Nine species were found. Eight species were identified morphologically; *Culicoides obsoletus*, *C. riethi*, *C. newsteadi*, *C. circumscriptus*, *C. festivipennis*, *C. punctatus*, *C. pictipennis* and *C. puncticollis*. The ninth species might be classified as either of *C. nubeculosus* or *C. riethi* and its identification needs additional investigations. The total prevalence of *Haemoproteus* in the examined biting midges was 2.17%. Three individuals of *C. pictipennis* were infected with the *Haemoproteus* lineage TURDUS2 (prevalence 16.67%), a common parasite of thrushes (Turdidae). Two individuals of *C. circumscriptus* contained *Haemoproteus* lineages (prevalence 2.78%); these were the lineage HAWF2 (previously reported from *Coccothraustes coccothraustes*) and a new lineage CULCIR1 not previously reported in the literature.

Keywords

Culicoides biting midges, *Haemoproteus*, specificity, vector-parasite interactions.

Introduction

Invasive pathogens, also known as agents of Emerging Infectious Diseases (EID), constitute a growing threat to biodiversity, livestock and human health (Daszak *et al*. 2000). Most of pathogens are able to infect a limited number of host species and, therefore, the geographical ranges of pathogen species are ultimately restricted by the range of their host. At present, the geographical ranges of many species are changing at unprecedented rates as a result of habitat alterations, accidental introduction of invasive species, global warming and other factors (Walther *et al*. 2002). Haemosporidian parasites are responsible for emerging diseases in humans as well as in domestic and wild animals (Valkiūnas 2005, Enayati and Hemingway 2010); this is a process becoming more relevant in the face of environmental changes. The warming climate accelerates the geographic expansion of both parasites and vectors into regions where they were previously absent (Jones *et al.* 2008). Despite its relevance for human and animal health, knowledge on the insect vectors transmitting haemosporidians is poor (Valkiūnas 2005).

Pigment-forming haemosporidian parasites (Haemosporida) of the genera *Haemoproteus* and *Plasmodium* are widespread in birds on all continents except Antarctica. These pathogens are transmitted exclusively by blood-sucking dipteran insects and are responsible for diseases, sometimes deadly, both in domestic and wild birds (Garnham 1966; Valkiūnas 2005). In order to understand evolutionary biology of haemosporidians, it is essential to identify where these parasites are transmitted and how the local diversity of the parasites varies across large geographical regions.

The majority of the avian *Haemoproteus* species are transmitted by biting midges of the genus *Culicoides*; also, hippoboscid flies (Hippoboscidae) act as vectors of these parasites (Garnham 1966; Valkiūnas 2005). However, it remains unknown which species of *Culicoides* transmit certain haemo-

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proteids, both at level of species and genetic lineages. For the first time, Martínez-de la Puente *et al*. (2011) used molecular methods and determined numerous associations between *Culicoides* spp. and avian *Haemoproteus* spp.; they revealed that there were both species-specific parasites and generalists among the *Haemoproteus* lineages relative to their vectors. Synek *et al*. (2013) showed variations in the levels of host specificity; they found one *Haemoproteus* lineage in several species of *Culicoides* and several *Haemoproteus* lineages occurring only in a single species of this dipteran genus.

The identification of *Culicoides* spp. is routinely carried out on the basis of morphological characters; these mainly refer to the wing pattern, the form of the male and female reproductive organs or characteristics of certain palpal segments (Delécolle 1985). The reliable identification of *Culicoides* spp. based on morphological characters requires taxonomic expertise and time-consuming preparation of midge individuals. The midges of the numerically largest pools in light trap catches, such as the *C. obsoletus* complex (*C. obsoletus sensu stricto* and *C. scoticus*), along with the morphologically similar *C. chiopterus* and *C. dewulfi* or the *C. pulicaris* complex (*C. pulicaris sensu stricto*, *C. punctatus*, *C. impunctatus*, *C. newsteadi*, *C. delta* and *C. lupicaris*) (EFSA 2008), are only 1 to 2 mm long and they are very similar in their morphology. Therefore, the rapid and reliable identification of large sample of midges is virtually impossible.

A molecular-genetic method based on the development of species-specific primers offers the opportunity of simplifying the identification process of the *Culicoides* midges (Nolan et *al*. 2007; Stephan *et al*. 2009). Numerous studies (Dallas *et al*. 2003; Li *et al*. 2003; Cêtre-Sossah *et al*. 2004; Wörheide *et al*. 2004; Perrin *et al*. 2006) dealing with a similar problem found the noncoding genetic region of the internal transcribed spacer 1 (ITS-1) of the mitochondrial ribosomal DNA and mitochondrial cytochrome *c* oxidase subunit 1 (COI) to be highly promising in order to establish a reliable polymerase chain reaction (PCR)-based method for species identification.

Up to this moment, 36 species of biting midges have been reported from Bulgaria (Appendix 1). All these species have been recorded on the basis of morphological identification. All previous studies of the genus *Culicoides* in Bulgaria were focused on their role in the transmission of the bluetongue virus (BTV), a disease of major economical importance (Purse *et al*. 2006; Nedelchev 2008).

The bird community in the region of Kalimok Biological Station (NE Bulgaria) has been intensely studied for the last 15 years. The prevalence and species diversity of haemosporidian parasites in birds as well as the number of *Haemoproteus* species with local transmission have been described for the same territory (Valkiūnas *et al*. 1999, 2007, 2008; Shurulinkov and Golemanski 2002, 2003, Zehtindjiev *et al*. 2008, Shurulinkov and Ilieva 2009, Dimitrov *et al*. 2010). The present study describes the species of biting midges in the same area of NE Bulgaria throughout the whole active season combining morphological and molecular methods for identification of species. In addition, we test for the potential role of the species recorded for the transmission of certain haemosporidian parasites of birds.

Materials and Methods

We collected *Culicoides* midges during May–October 2011 at Kalimok Biological Station (44°00′N, 26°26′E, Silistra District) using a UV-light/suction trap (OVI) manufactured by the Onderstepoort Veterinary Institute (Republic of South Africa) (Venter and Meiswinkel 1994). A trap consisting of an 8 W UV-light bulbs and a down-draught suction motors, powered by a car battery, was placed on a tree at a height of 1.5–2.0 m above the ground near two habitats, i.e. reedbeds of *Phragmites australis*, partly surrounded by dwarf elder (*Sambucus ebulus*) and deciduous forest of false acacia (*Robinia pseudoacacia*) and raywood ash (*Fraxinus oxycarpa*). Trapping was carried out during sunset for 2 hours before sunset and lasted approximately 4 hours every day with suitable weather conditions. We had 121 sampling nights and yielded 230 parous (Dyce 1969) female specimens of biting midges. Samples were subsequently processed in the lab under a stereomicroscope.

Morphological identification of Culicoides spp.

All *Culicoides* specimens were preserved in 70% ethanol. The identification was processed according to their wing pattern as observed with an Olympus CZ51 stereomicroscope using the keys by Chvála (1980) and Glukhova (1989). In addition, for more accurate identification, 3 specimens of each species were dissected and the head, wings and posterior abdominal segments of females were mounted on microscope slides using CMCP-10 mounting media (Polysciences Inc., Warrington, PA, USA) and preserved in the collection of the Institute of Biodiversity and Ecosystem Research, Sofia.

PCR protocol for identification of Culicoides spp.

Female *Culicoides* individuals were homogenized into 1.5 ml tubes with 100 µl of lysis buffer (0.1 M Tris, 0.005 M EDTA, 0.2% SDS, 0.2 M NaCl, pH 8.5) and 1.5 µl proteinase K (20 mg/ml) and incubated for 3 hours at 56°C. After the incubation, the samples were centrifuged for 10 min at 10,000 rpm. The supernatant was replaced into new tubes with 10 µl of NaAc (3M) and add 220 µl (two volumes) of ice cold 95% ethanol and centrifuged again for 10 min at 10,000 rpm. The supernatant was removed and the pellet was washed with 100 µl ice cold 70% ethanol and centrifuged for 5 min at 10,000 rpm. The ethanol was removed and the pellet was let to dry in open tubes overnight.

The DNA was diluted to a standard concentration of 25 ng/µl for PCR. Diluted genomic DNA was used as a template for amplification of a fragment of mitochondrial cytochrome *c* oxidase subunit I gene (COI).

The initial PCR activation step for the used C1-J-1718-mod (GGWGGRTTTGGWAAYTGAYTAG) and C1-N-2191-

Table I. Species of the genus *Culicoides* recorded from the area of Kalimok Biological Station, NE Bulgaria, in the active season of 2011

Species	Number of individuals	Identification method
C.circumscriptus	72	Morphologically; PCR and sequencing
C.festivipennis	32	Morphologically; PCR and sequencing
C. newsteadi	2	Morphologically; PCR and sequencing
C. obsoletus	4	Multiple PCR (Nolan *et al*. 2007)
C. pictipennis	18	Morphologically; PCR and sequencing
C. punctatus	25	Morphologically; PCR and sequencing
C. puncticollis	26	Morphologically; PCR and sequencing
C. riethi	31	Morphologically; PCR and sequencing
**Culicoides* sp.	20	Morphologically; PCR and sequencing

*The morphological approach identified this species as *C. nubeculosus* but the molecular identification does not correspond to the same species. Sequence analysis shows 4 mismatching nucleotides between *Culicoides* sp. and *C. riethi*.

mod (AGHWCCAAAAGTTTCYTTTTTCC) primer pair, modified from previously described primers (Dallas *et al*. 2003), was performed at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 30 sec. The final cycle was completed with 10 min of extension at 72°C. Standard sequencing of the 585 bp amplicons was performed by Macrogen Ltd for 3 representatives of each species. Obtained sequences were edited using BioEdit (Hall 1999) and compared with the available sequences in the GenBank DNA data-

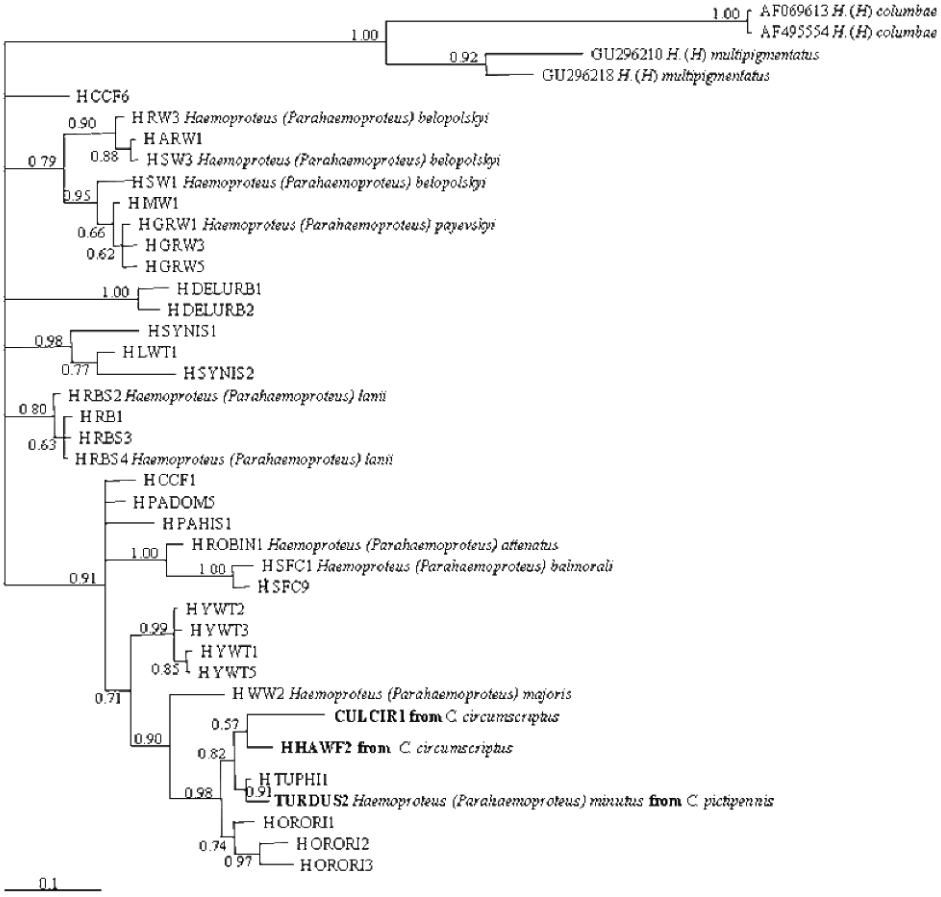

Fig. 1. Bayesian phylogeny of 40 cytochrome *b* gene lineages of *Haemoproteus* spp. reported from the study area. *H. columbae* and *H. multipigmentatus* were used as an outgroup. Lineages recorded in our study in *Culicoides* spp. are given in bold. Codes of the lineages (when available) are given before the species names of parasites. Nodal support values near branches indicate posterior clade probabilities.

base. All positive for haemosporidians biting midges were sequenced in order to confirm the morphological identification.

PCR protocol for identification of haemosporidian parasites

Diluted genomic DNA was used as the template in a PCR assays for detection of parasites using primers and temperature profiles as in Hellgren *et al*. (2004) and Waldenström *et al.* (2004). Positive or negative samples were seen as presence or absence of bands on a 2% agarose gel using 2.5 µl of the final PCR product. Amplified fragments were sequenced from the 5' end with the primer HaemF (ATGGTGCTTTCGATATATGCATG) in Macrogen Ltd. The obtained sequences of 479 bp of mitochondrial cytochrome *b* gene were edited, aligned and compared in a sequence identity matrix using the program BioEdit (Hall 1999). All unique lineages, i.e. sequences differing by one or more nucleotide difference, were sequenced in the reversed direction with the complementary primer. The obtained sequences were matched and named according to the MalAvi Database (Bensch *et al*. 2009).

Statistical analyses

The analysis of evolutionary history was performed using MrBayes version 3.1.2. (Ronquist and Huelsenbeck 2003). We used General Time Reversible model including invariable sites and variation among sites (GTR+I+G) as suggested by the software MrModeltest 2.2 (Nylander 2004; software available from <http://www.ebc.uu.se/systzoo/staff/nylander.html>). Two simultaneous runs were conducted with a sample frequency of every 100th generation over 7 million generations. The 25% of the trees were discarded as burn-in period. The remaining trees were used to construct a majority rule consensus tree. The phylogenies were visualized using Tree View 1.6.6.

Results

We collected and identified 230 individuals from the area of Kalimok Biological Station (NE Bulgaria), which belonged to 9 species of the genus *Culicoides* (Table I). One of these species was identified as *C. nubeculosus* on the basis of morphological characters; however, the sequence analysis showed 55 mismatching nucleotides with *C. nubeculosus* and 4 mismatching nucleotides with *C. riethi.* Therefore, the identification of this species needs additional investigations.

All specimens were checked for the presence of haemosporidians. Two out of 72 individuals of *Culicoides circumscriptus* were infected with avian *Haemoproteus* lineages (prevalence 2.78%). One of the parasites was identified as the lineage HAWF2. The second was identified as a new lineage exhibiting more than 98% match with a *Haemoproteus* sp. parasite previously found in the American crow *Corvus brachyrhynchos* (Fig. 1). The new lineage is named CULCIR1 and it is submitted in GenBank with accession No. KC815468. Three individuals of *Culicoides pictipennis* were infected with the *Haemoproteus* lineage TURDUS2 (prevalence of 16.67%). The total prevalence of *Haemoproteus* lineages in all nine species studied was 2.17%.

Discussion

The recorded number of *Culicoides* spp. in our study adds to the previous knowledge of this group of important blood sucking insects of humans and livestock. Many *Culicoides* spp. are vectors of BTV (Reoviridae: *Orbivirus*), a virus that can replicate in any ruminant, first detected in Central Europe in 2006 (Mehlhorn *et al*. 2008). *C. obsoletus*, *C. pulicaris* and *C. scoticus* are considered the main vectors of BTV in Europe and their bloodmeals derived only from livestock (Mehlhorn *et al.* 2007, Bartsch *et al*. 2009). Santiago-Alarcon *et al*. (2012) found *C. obsoletus*, which has previously been found in Germany, and together with *C. scoticus* and *C. pulicaris*, has been feeding mainly on humans. As BTV is already established in many countries it can potentially invade humans via infectious *Culicoides* bites; however this virus apparently does not cause acute illness in humans (Santiago-Alarcon *et al*. 2012).

Because of the potential role of these vectors in the BTV transmission, we have to consider all available information for the studied territory. Two epizooties of bluetongue virus (serotype 9) were observed in Bulgaria in 1999 and 2001 (Purse *et al*. 2006). The following species are related to the bluetongue disease: *Culicoides pulicaris* complex, *C. obsoletus* complex and *Monoculicoides* group (see Appendix 1) (Nedelchev 2008). A survey focused on the culicoid vectors of BTV in Bulgaria (Purse *et al*. 2006) revealed *C. obsoletus* complex as the most abundant followed by *C. pulicaris* complex; these two complexes represented 75% and 16%, respectively, of all individuals trapped, whereas, the major Old World vector, *C. imicola*, was not found. In our study, the representatives of subgenus *Monoculicoides* (*C. puncticollis*, *C. nubeculosus* and probably *C. riethi*) were the most abundant (33.48%) followed by the ornithophilic *C. circumscriptus* representing 31.30% of the insects sampled. These differences in the species composition shown in our study may be due to the lack of livestock and the habitat characteristics in the studied area.

The list of biting midge species recorded in our study represents 25% of the species previously reported from the territory of Bulgaria. The present study provides the first data on the prevalence of haemosporidian infections and the species identity of one *Haemoproteus* lineage associated with *Culicoides* midges in Bulgaria. Several studies have shown that some *Culicoides* spp. have ornithophilic food preferences (Martínez-De La Puente *et al*. 2011, Pettersson *et al*. 2012, Santiago-Alarcon *et al*. 2012). According to these publications, the Bulgarian list of species includes five species feeding on birds (Appendix 1). In our study, we found four of these (*C. circumscriptus*, *C. punctatus*, *C. pictipennis* and *C. fes-*

Appendix 1. Checklist of the genus *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae), recorded in Bulgaria (Zilahi 1934, Remm 1988, Nedelchev 2008). For each species it is indicated (+) whether found in the current study, reported to be ornithophilous and vectors of bluetongue virus

***Culicoides* spp. reported from Bulgaria**	**Ornithophilous**	**Recorded in the current study**	**Vectors of bluetongue virus**
Culicoides circumscriptus Kieffer, 1918	+	+	
Culicoides deltus Edwards, 1939			+
Culicoides dewulfi Goetghebuer, 1936	+		+
Culicoides fagineus Edwards, 1939			
Culicoides fascipennis (Staeger, 1839)			
Culicoides festivipennis Kieffer, 1914	+		+
Culicoides flavipulicaris Dzhafarov, 1964			
Culicoides gejgelensis Dzhafarov, 1964			
Culicoides grisescens Edwards, 1939			
Culicoides impunctatus Goetghebuer, 1920			+
Culicoides kurensis Dzhafarov, 1960			
Culicoides longipennis Khalaf, 1957			
Culicoides newsteadi Austen, 1921		+	+
Culicoides nubeculosus (Meigen, 1830)		+	+
Culicoides obsoletus (Meigen, 1818)	+	+	+
Culicoides odiatus Austen, 1921			
Culicoides ostroushkoae Glukhova, 1989			
Culicoides pallidicornis Kieffer, 1919			
Culicoides parroti Kieffer, 1922			+
Culicoides pictipennis (Staeger, 1839)	+	+	
Culicoides pulicaris Linnaeus, 1758			+
Culicoides punctatus Meigen, 1804	+	+	+
Culicoides puncticollis (Becker, 1903)		+	+
Culicoides reconditus Campbell and Pelham-Clinton, 1960	+		
Culicoides riethi Kieffer, 1914		+	+
Culicoides saevus Kieffer, 1922			
Culicoides salinarius Kieffer, 1914	+		
Culicoides schultzei Enderlein, 1908			
Culicoides scoticus Downes and Kettle, 1952	+		+
Culicoides sejfadinei Dzhafarov, 1958			
Culicoides shaklawensis Khalaf, 1957			
Culicoides simulator Edwards, 1939	+		
Culicoides stigma (Meigen, 1818)			+
Culicoides subfasciipennis Kieffer, 1919			
Culicoides tauricus Gutsevich,1959			
Culicoides vexans (Staeger, 1839)			

tivipennis) in the territory studied, which potentially can be responsible for the transmission of *Haemoproteus* spp. in the region.

Currently, it is widely accepted that vectors of certain dipteran families transmit certain haemosporidians (Valkiūnas 2005, Martinsen *et al*. 2008). For example, avian haemosporidians of the genera *Haemoproteus* (subgenus *Parahaemoproteus*) and *Leucocytozoon* (subgenus *Akiba*) are considered to be transmitted only by *Culicoides* vectors (Valkiūnas 2005, Martinsen *et al*. 2008). Associations between various *Culicoides* spp. (*C. circumscriptus*, *C. pictipennis*, *C. festivipennis*, *C. salinarius*, *C. duddingstoni*, *C. kibunensis*, *C. punctatus*, *C. simulator* and *C. segnis*) and *Haemoproteus* lineages have been already published before (Martínez-De La Puente *et al*. 2011, Santiago-Alarcon *et al*. 2012, Synek *et al*. 2013). In our study, we have found three lineages of *Haemoproteus* infecting two species of *Culicoides*. Despite of the potential possibility that the recorded lineages can only have abortive development in the related *Culicoides* spp. (Valkiūnas 2011), it is important to note that HAWF2 lineages of *Haemoproteus* is successfully transmitted in the same territory studied before concerning haemosporidian parasites (Dimitrov *et al*. 2010). Hence the established vectors are suspected to be responsible for transmission of these parasites in the region.

The hypothesis (Fig. 1) about the phylogenetic relations between the all haemoproteus lineages reported from the same territory and those found in the two different vectors in our study indicate high specialisation of the parasites to their final

hosts. The lineages CULCIR1 and HAWF2 cluster together and are found in *C. circumscriptus* while the third found lineage TURDUS2 (*Haemoproteus minutus*) belongs to another clade and is transmitted by another insect according to our data – *C. pictipennis.* TURDUS2 has been previously found in the same *Culicoides* species (Santiago-Alarcon *et al*. 2012) and recently has been detected in *C. kibunensis* and *C. festivipennis* (Synek *et al*. 2013).

This information is important for future epidemiological studies and better understanding of avian disease transmission, particularly in the light of recent discoveries on the pathogenicity of *Haemoproteus* spp. in avian hosts (Ferrell *et al*. 2007; Olias *et al*. 2011). However, because of the complicated life cycles of haemosporidians, it should be pointed out that vector interpretations of this and similar studies need validation because PCR amplifies haemosporidian DNA regardless of the life stages present in these biting insects (Valkiūnas 2011). In this relation, an important issue of haemosporidian vector research is the need for increased collaboration between researchers from different fields.

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