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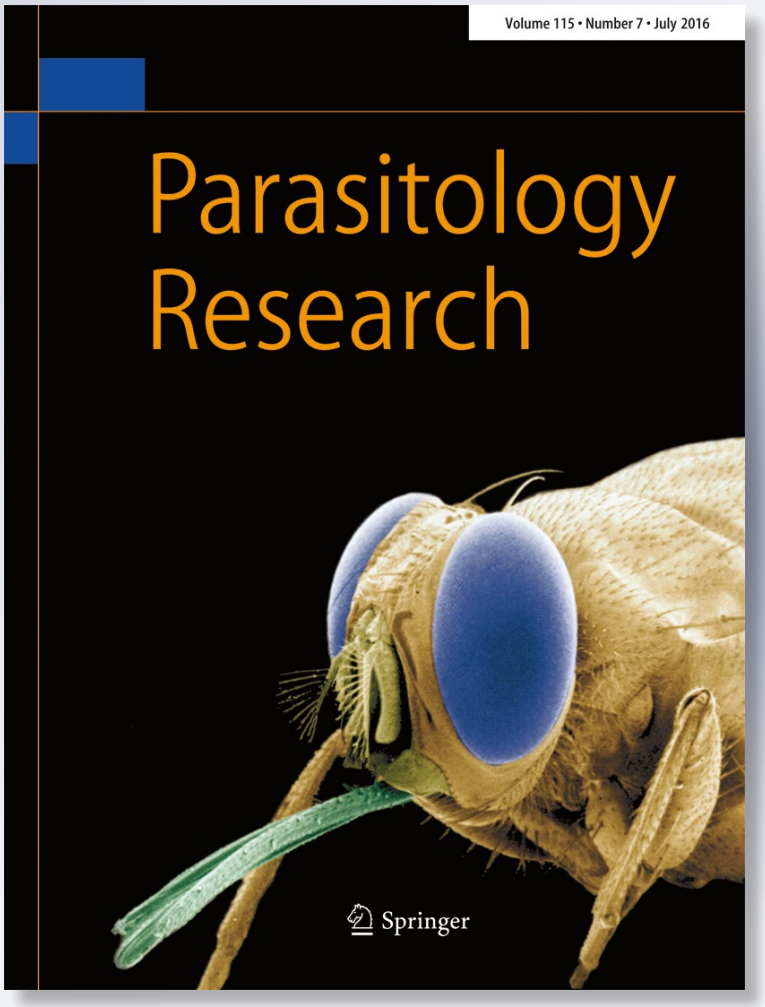

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ORIGINAL PAPER

Description of *Haemoproteus ciconiae* sp. nov. (Haemoproteidae, Haemosporida) from the white stork *Ciconia ciconia*, with remarks on insensitivity of established polymerase chain reaction assays to detect this infection

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Abstract *Haemoproteus ciconiae* sp. nov. (Haemosporida, Haemoproteidae) was found in the white stork *Ciconia ciconia* (Ciconiiformes, Ciconiidae) after spring migration in Lithuania. This organism is illustrated and described based on the morphology of its blood stages. The new species can be readily distinguished from all other haemoproteids parasitizing ciconiiform birds due to the presence of large number (approximately 20 on average) small (<1 μm) pigment granules in its mature gametocytes. Growing and mature gametocytes of *H. ciconiae* were readily visible in all blood films (parasitemia of 0.001 %). However, experienced researchers were unable to detect sequences of its mitochondrial cytochrome *b* (cyt *b*) or apicoplast genes from the microscopically positive sample by using five established assays for polymerase chain reaction (PCR)-based detection of avian haemosporidian parasites. The white stork cyt *b* sequence was readily detectable, indicating the well-optimised PCR protocols and the good quality of total DNA in the sample containing the new species. The failure to amplify this parasite DNA indicates insufficient sensitivity of the currently used PCR-based assays in diagnostics of avian haemosporidian infections. We suggest possible explanations of this observation. To minimize number of the false negative PCR reports, we call for the continued use of optical microscopy in parallel with molecular diagnostics in studies of haemosporidian parasites, particularly in wildlife.

Keywords *Haemoproteus* · New species · Birds · *Cicocia ciconia* · PCR · Optical microscopy

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Introduction

The white stork *Ciconia ciconia* is a widespread Palearctic bird belonging to the Ciconiidae (order Ciconiiformes) (Cramp and Simmons 1977). This common big bird is well-recognisable and often considered as an indicator of ecologically healthy environment in agricultural landscapes where it breeds singly in big solitary trees, on roofs of houses and on telegraph poles. Currently, population of the white stork is increasing throughout its entire breeding range, particularly markedly in the Eastern part of Europe, probably due to changes in farming practices and foraging conditions at wintering areas (Vaitkuvienė and Dagys 2015). North European population of the white stork winters in Africa where these birds spend the great part of a year and are exposed to parasite infections. However, haemosporidians (Haemosporida) have not been reported in the white stork in Europe (Valkiūnas et al. 2002), but were described in other species of ciconiiform birds in sub-Saharan Africa (Tendeiro 1947; Peirce and Cooper 1977; Valkiūnas 2005). It remains unclear if white storks are infected with blood parasite at African breeding grounds and if they transport these infections to European breeding areas.

In spring 2015, one damaged adult white stork was delivered to the Ventės Ragas Ornithological Station, in Lithuania. Blood samples were collected and examined by microscopic examination of blood films and polymerase chain reaction (PCR)-based methods. One previously undescribed species of *Haemoproteus* (Haemoproteidae, Haemosporida) was reported in blood films, but it was undetectable by five well-established PCR assays. This parasite is named and described here based on morphology of its blood stages. It is the first

haemosporidian species described from the white stork (Bennett et al. 1982; Bishop and Bennett 1992; Valkiūnas 2005). We compare the new species with haemoproteids reported in other ciconiiform birds and discuss failures in detection of its DNA sequences by the currently broadly used PCR-based protocols.

Materials and methods

Study site, collection of samples and their microscopic examination

On 20 May 2015, one female of the white stork with both broken legs was found in Šilutė District of Lithuania and delivered to the Ventės Ragas Ornithological Station. Blood was taken by puncturing the brachial vein. A blood sample (~50 μL) was collected using heparinized microcapillaries and stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) at ambient temperature while in the field and then preserved at −20 °C in the laboratory. We prepared 10 blood films on ready-to-use glass slides and air-dried them within 5 s after preparation using a battery-operated fan. The blood films were fixed in absolute methanol for 1 min on the day of their preparation. The fixed smears were air-dried and stained in a 10 % working solution of a commercially purchased stock solution of Giemsa stain for 1 h. All blood films were stained within 14 days after their fixation. Details of preparation and staining of blood films were described by Valkiūnas et al. (2008).

The white stork was markedly atonic, and the veterinary observation concluded that it is impossible to treat due to the marked leg damage and weak body condition. The bird was euthanized, and pieces of organs (liver, lungs, heart, kidneys, bone marrow) and pectoral muscles were fixed in 10 % neutral formalin, embedded in paraffin and processed using traditional histology methods (Valkiūnas 2005). Histological sections of 4 μm were obtained, stained with hematoxylin-eosin, mounted in BioMount (BioGnost, Croatia) and examined microscopically.

An Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE was used to examine blood films and histological preparations, to prepare illustrations and to take measurements. Approximately 150 fields were examined at low magnification (×400), and then at least 100 fields were viewed studied at high magnification (×1000). The morphometric features studied (Table 1) are those defined by Valkiūnas (2005). Intensity of parasitemia was estimated as a percentage by counting of the number of parasites per 100,000 red blood cells. The morphometric analysis (Table 1) was carried out using the 'Statistica 7' package.

Table 1 Morphometry of host cells and mature gametocytes of *Haemoproteus ciconiae* sp. nov. from the white stork *Ciconia ciconia*

Feature	Measurements (μm)[a]
Uninfected erythrocyte	
Length	13.3–15.3 (14.5 ± 0.6)
Width	6.4–7.9 (7.4 ± 0.4)
Area	76.9–95.5 (85.2 ± 5.9)
Uninfected erythrocyte nucleus	
Length	5.6–7.5 (6.5 ± 0.5)
Width	1.9–2.8 (2.4 ± 0.2)
Area	10.3–15.5 (12.7 ± 1.2)
Macrogametocyte	
Infected erythrocyte	
Length	14.3–17.5 (15.9 ± 0.8)
Width	5.7–8.7 (7.4 ± 0.8)
Area	82.3–108.5 (95.3 ± 7.5)
Infected erythrocyte nucleus	
Length	5.7–8.5 (6.9 ± 0.6)
Width	1.7–2.7 (2.2 ± 0.3)
Area	9.2–17.5 (12.4 ± 1.9)
Gametocyte	
Length	11.5–16.8 (14.1 ± 1.3)
Width	2.4–3.8 (3.0 ± 0.4)
Area	32.7–51.7 (38.3 ± 4.3)
Gametocyte nucleus	
Length	2.4–4.7 (3.3 ± 0.8)
Width	0.8–3.1 (1.9 ± 0.5)
Area	3.0–9.0 (5.1 ± 1.7)
Pigment granules	20.0–29.0 (23.7 ± 2.3)
NDR[b]	0.4–1.0 (0.7 ± 0.2)
Microgametocyte	
Infected erythrocyte	
Length	13.7–18.7 (16.3 ± 1.1)
Width	6.8–8.7 (7.6 ± 0.5)
Area	83.1–116.7 (101.3 ± 9.0)
Infected erythrocyte nucleus	
Length	6.2–7.7 (7.0 ± 0.4)
Width	1.8–3.1 (2.4 ± 0.3)
Area	9.2–19.5 (14.0 ± 2.7)
Gametocyte	
Length	12.7–18.8 (15.6 ± 1.7)
Width	2.2–4.0 (3.0 ± 0.5)
Area	35.5–58.6 (46.2 ± 6.0)
Gametocyte nucleus	
Length	–
Width	–
Area	–
Pigment granules	11.0–23.0 (18.1 ± 2.6)
NDR	0.3–1.0 (0.7 ± 0.2)

[a] All measurements (n = 21) are given in micrometers. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation

[b] NDR = nucleus displacement ration, which was calculated according to Bennett and Campbell (1972)

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from blood samples using the standard ammonium-acetate protocol (Sambrook and Russell 2001) with minor modification: instead of 250 μL of fixed blood, we used 125 μL. Quantification of the DNA was performed by using a NanoDrop spectrophotometer (Implen NanoPhotometer P330). All reactions were performed in

25 μL total volumes, including 50 ng of a total genomic DNA template (2 μL), 12.5 μL of DreamTaq Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 μL nuclease-free water and 1 μL of each primer. One negative control (nuclease-free water) and one positive control (a blood sample, which was positive by microscopic examination of blood films) were used to control for false amplifications. All used PCR essays (Table 2) correctly determined both these control samples. If the white stork blood sample was classified as a false negative, we tested it at least three times by each PCR essay to ensure that the negative results were not caused by the PCR failure.

Five established PCR assays (Table 2), which are broadly used in wildlife avian haemosporidian research, were applied for detection of *Haemoproteus* infection from the white stork blood sample. This sample was *Haemoproteus* parasite positive by microscopic examination of blood films. Four of these PCR assays used primer sets, which amplify fragments of the mitochondrial genome: three of cytochrome *b* (cyt *b*) gene (primer set HaemNFI/NR3 and HAEMF/R2 (Bensch et al. 2000; Hellgren et al. 2004), primers 621F/983R (Richard et al. 2002), and primers 3760F/4292Rw2 (Beadell et al. 2004)), one of cytochrome oxidase subunit I (COI) gene (primer set COIF/R and COIF2/R2, Martinsen et al. 2008) and one target apicoplast genome (primer set ClpcF/R and ClpcF2/R2, Martinsen et al. 2008). Because all these PCR essays were insensitive in detection of *Haemoproteus* infection in the microscopy positive blood sample (see below), we ruled out a possibility of insufficient quality of the total DNA in our sample by application of primers, which amplify fragments of cyt *b* gene of birds (primers Avian-3 and Avian-8, Ejiri et al. 2011).

The amplification success was evaluated after runout of 2 μL of final PCR product on 2 % agarose gels. PCR products (21 μL) were purified by adding 11 μL of NH_4Ac, 37 μL of 96 % and 150 μL of 70 % ethanol. After centrifugation, air-dried DNA pellets were dissolved in 15 μL of ddH_2O. To identify sequences, samples that showed positive amplification were sequenced using Big Dye Terminator V3.1 Cycle Sequencing Kit and then loaded on an ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, Foster City, California). The sequences were edited and aligned using BioEdit software (Hall, 1999). The BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare detected sequences with those deposited in GenBank.

Results

Microscopic examination revealed an undescribed species of *Haemoproteus* in the white stork. Intensity of parasitemia was 0.001 %. Over 100 gametocytes and parasitized red blood cells were examined. Exoerythrocytic meronts were not reported in histological sections of organs. Other blood parasites were not seen.

Table 2 Six polymerase chain reaction (PCR) assays used in detection of bird and haemosporidian parasite partial DNA sequences from blood samples

Gene	Primer code	Primer sequence	Reference
Apicoplast[a]	Outer ClpcF	5′-AAACTGAATTAGCAAAAATATTA-3′	Martinsen et al. 2008
	ClpcR	5′-CGWGCWCCATATAAAGGAT-3′	
	Inner ClpcF2	5′-GATTTGATATGAGTGAATATATGG-3′	
	ClpcR2	5′-CCATATAAAGGATTATAWG-3′	
Cytochrome oxidase subunit I[a]	Outer COIF	5′-CTATTTATGGTTTTCATTTTTATTTGGTA-3′	
	COIR	5′-AGGAATACGTCTAGGCATTACATTAAATCC-3′	
	Inner COIF2	5′-ATGATATTTACARTTCAYGGWATTATTATG-3′	
	COIR2	5′-GTATTTTCTCGTAATGTTTTACCAAAGAA-3′	
Cytochrome *b*[a]	Outer HaemNFI	5′-CATATATTAAGAGAAITATGGAG-3′;	Bensch et al. 2000;
	HaemNR3	5′-ATAGAAAGATAAGAAATACCATTC-3′	Hellgren et al. 2004
	Inner HAEMF	5′-ATGGTGCTTTCGATATATGCATG-3′	
	HAEMR2	5′-GCATTATCTGGATGTGATAATGGT-3′	
Cytochrome *b*	3760F	5′-GAGTGGATGGTGTTTTAGAT-3′	Beadell et al. 2004
	4292Rw2	5′-TGGAACAATATGTARAGGAGT-3′	
Cytochrome *b*	621F	5′-AAAAATACCCTTCTATCCAAATCT-3′	Richard et al. 2002
	983R	5′-CATCCAATCCATAATAAAGCAT-3′	
Cytochrome *b*	Avian-3	5′-GACTGTGAYAAAATYCCMTTCCA-3′	Ejiri et al. 2011
	Avian-8	5′-GYCTTCAITYTTTGGYTTACAAGAC-3′	

[a] Nested PCR

None of the applied PCR assays (Table 2) detected new *Haemoproteus* infection in the sample, which was microscopy positive. However, amplifications of (1) the control positive samples and (2) the bird DNA were successful, indicating the well-optimised PCR protocols and the good quality of the total DNA in the same sample containing the new species. The sequencing and application of the BLAST algorithm confirmed that we obtained bird cyt *b* sequence belonging to the white stork (GenBank accession no. U70822.1).

Parasite description

***Haemoproteus (Parahaemoproteus) ciconiae* n. sp. (Fig. 1, Table 1)**

Macrogametocytes (Fig. 1a–h, Table 1) Macrogametocytes develop in mature erythrocytes. Earliest forms were not seen in the type material. Growing gametocytes assume lateral positions to the nuclei of infected erythrocytes; they often do not touch either envelop or nuclei, or both these structures of the host cells (Fig. 1a). Cytoplasm is finely granular in appearance, sometimes contains several small vacuoles (Fig. 1e, g); volutin granules were not seen. Gametocytes extend along the nuclei of erythrocytes; they do not enclose or only slightly enclose the nuclei with their ends (Fig. 1a–d). Fully grown gametocytes do not fill the erythrocytes up to their poles (Fig. 1e–h); they have no permanent position in erythrocytes: gametocytes touching both the nuclei and envelop are common (Fig. 1g), but the parasites, which do not touch either envelop (Fig. 1e) or nuclei (Fig. 1f, h), are also present. Outline usually is entire (Fig. 1g, h), sometimes slightly wavy (Fig. 1f). Parasite nucleus (Fig. 1b, h) is compact, markedly variable in form, median or submedian in position; it has no single predominant position in relation to gametocyte pellicle (compare Fig. 1a, e). Nucleolus was not seen. Pigment granules (Fig. 1d, e) are mainly small (<0.5 μm), occasionally of medium (0.5–1.0 μm) size, roundish, usually randomly scattered throughout the cytoplasm (Fig. 1a, e–g), but also might be grouped (Fig. 1c, e, h). Nuclei of infected

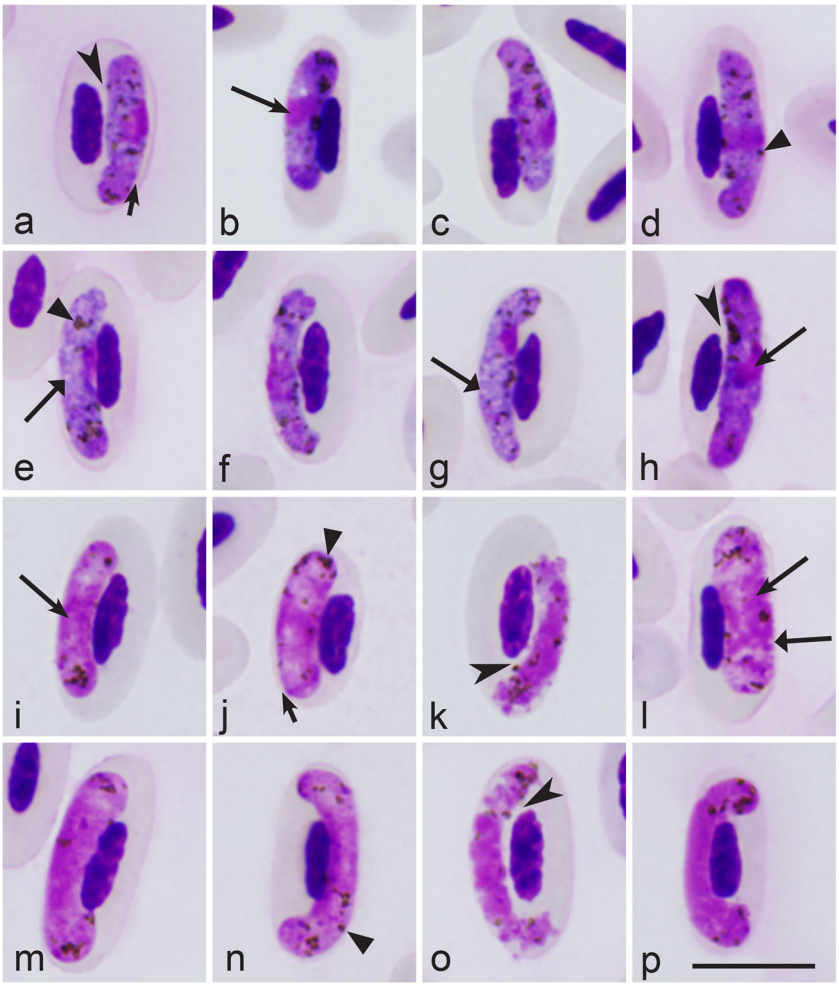

Fig. 1 Macrogametocytes (**a–h**) and microgametocytes (**i–p**) of *Haemoproteus ciconiae* sp. nov. from the blood of the white stork *Ciconia ciconia*. Giemsa-stained thin blood films. *Long simple arrows*—nuclei of parasites; *short simple arrows*—unfilled spaces between gametocytes and erythrocyte envelope; *simple arrowheads*—unfilled spaces between gametocytes and erythrocyte nuclei; *long triangle arrows*—vacuoles; *triangle arrowheads*—pigment granules. Note that length of mature macrogametocytes is less than in mature microgametocytes (compare **f–h** with **n–p**). *Scale bar* = 10 μm

erythrocytes are not displaced (Fig. 1f, g) or are slightly (Fig. 1a–e, h) displaced laterally. Parasitized erythrocytes are enlarged in length (Table 1).

Microgametocytes (Fig. 1i–p, Table 1) The general configuration is as for macrogametocytes with the usual haemosporidian sexually dimorphic characters, which are the pale staining of the cytoplasm and large diffuse nuclei. Boundaries of the parasite nuclei are irregular and obscure (Fig. 1i–p), making the nuclei difficult to measure. Gametocyte outline varies from complete even (Fig. 1i, j, l, m, n, p) to highly amoeboid (Fig. 1k, o). Fully grown microgametocytes are larger than macrogametocytes (Table 1), and they slightly enclose erythrocyte nuclei with their ends (Fig. 1n, p). Gametocytes with highly amoeboid outline are present. Other characters are as for macrogametocytes.

Taxonomic summary

Type host White stork *Ciconia ciconia* L. (Ciconiiformes, Ciconiidae)

Type locality Ventės Ragas, Lithuania (55° 20′ 28.1″ N, 21° 11′ 25.3″ E, 1 m above sea level)

Site of infection Mature erythrocytes; no other data

Type specimens Hapantotype (accession nos. 48912–48915 NS, intensity of parasitemia is 0.001 %, *Ciconia ciconia*, Ventės Ragas, collected by M. Ilgūnas, 20 May 2015) was deposited in the Nature Research Centre (NRC), Vilnius, Lithuania. Parahapantotypes (accession nos. G466178 and G466179, other data as for the hapantotype) were deposited in the Queensland Museum, Queensland, Australia. Parasite gametocytes were marked with circles on the hapantotype and parahapantotype slides.

Additional material Voucher blood films (accession nos. 48916–48919 NS) and the sample of whole blood from the type host (original field number 211/15R) were deposited in NRC, Vilnius, Lithuania.

Etymology The species name is derived from the host genus *Ciconia*, to which the type host of the parasite belongs.

Taxonomic remarks Four species of *Haemoproteus* parasitize birds belonging to the order Ciconiiformes: *H. crumenium* (Peirce 1987; Valkiūnas 2005), *H. herodiadis* (de Mello 1935a; Valkiūnas 2005), *H. plataleae* (de Mello 1935b; Bennett et al. 1975) and *H. pelouroi* (Tendeiro 1947; Valkiūnas 2005). These species can be distinguished from *H. ciconiae* due to the following readily discernable characters. In *H. crumenium,* macrogametocyte nuclei assume strictly central position and they adhere to the nuclei of infected erythrocytes. Additionally, the cytoplasm often contains prominent circular vacuoles. In *H. plataleae*, the number of pigment granules in macrogametocytes is approximately half as many in microgametocytes. In *H. herodiadis,* pigment granules are not numerous (approximately 10 on average), small (<0.5 μm) and dust-like in appearance. In *H. pelouroi*, both macrogametocytes and microgametocytes are markedly amoeboid and possess about ten pigment granules on average. None of these features are characters of *H. ciconiae* (Fig. 1, Table 1).

Among the haemoproteids of ciconiiform birds, *H. ciconiae* is most similar to *H. herodiadis*, and it can be distinguished from the latter species, primarily on the basis of (1) the greater number of pigment granules and (2) the larger size of pigment granules in its gametocytes.

Discussion

The key result of this study is the description of the first *Haemoproteus* parasite inhabiting the white stork. One may ask whether it is justifiable to describe a new species of *Haemoproteus* on the basis of a single set of blood films prepared from one individual host. Several valid species of haemosporidians with readily distinct morphological characters have been described on single records in naturally infected reptiles, birds and mammals (Garnham 1966; Valkiūnas 2005; Telford 2009; Zehtindjiev et al. 2012). The morphological characters of blood stages of *H. ciconiae* are distinct to differentiate this parasite from other known *Haemoproteus* species. The recorded parasites and their host cells were consistent in morphology and with the interpretation that they belong to *H. ciconiae*. The type material is deposited in two museums and is available for taxonomic examination. Importantly, the original sample of whole blood from the type host also is available for future examination, thus this study is readily repeatable. Because of difficulties in blood sampling from adult white storks (see below), this information might be lost if the new species is not described.

Avian *Haemoproteus* parasites belong to two subgenera, i.e. *Parahaemoproteus* and *Haemoproteus*. Traditional subgenetic classification of these haemoproteids is based mainly on the differences in their sporogony and distribution by vectors and avian hosts (Garnham 1966; Atkinson et al. 2008; Valkiūnas 2015). Sporogony of *Parahaemoproteus* spp. occurs in biting midges (Ceratopogonidae). These parasites inhabit mainly terrestrial birds of numerous avian orders, including ciconiiform birds, as is the case with *H. ciconiae*. Species of *Haemoproteus* have been reported mainly in birds belonging to the order Columbiformes and some marine birds

(Charadriiformes, Pelecaniformes); they are transmitted by louse flies of the Hippoboscidae (Levin et al. 2012; Merino et al. 2012). Vectors of *H. ciconiae* remain unknown. Phylogenies based on mitochondrial gene sequences can be used for prediction of vectors of *Haemoproteus* parasites (Bukauskaitė et al. 2015); however, the sequence information about *H. ciconiae* currently is absent. We attribute this parasite to subgenus *Parahaemoproteus* due to its ability to parasitize birds of the Ciconiiformes. However, investigation of sporogony is needed to prove subgeneric classification of this parasite.

In spite of their wide distribution, adult white storks are difficult to catch using traditional ornithological methods of netting and trapping, resulting in a few studies addressing its blood parasites (Rousselot 1953; Berson 1964; Jovani et al. 2001). There is no information about blood parasites of this bird species in Europe (Bennett et al. 1982; Bishop and Bennett 1992; Jovani et al. 2001; Valkiūnas 2005). Valkiūnas et al. (2002) collected and examined blood samples from 22 nestlings of the white stork on the Curonian Spit in the Baltic Sea. The birds were taken from nests at the age of approximately 5 weeks during implementation of an ornithological migration program and raised by hand in the vector-opened aviary. The blood of these birds was sampled and examined microscopically twice when the nestlings were 6 and 8 weeks old. Blood parasites were not reported. Absence of *Haemoproteus* parasites in juvenile birds indicates that white storks get infected with *H. ciconiae* away from breeding areas and bring this parasite to Europe, but transmission does not occur. The obstacles for transmission might be insufficient abiotic conditions for development of the African origin parasite in the northern Europe or lack of susceptible vector species, or both. Experimental studies are needed to answer these questions.

Description of new species is in accord with the recent PCR-based research showing remarkable genetic diversity of avian haemosporidian parasites (Bensch et al. 2004; Clark et al. 2014; Perkins 2014; Outlaw and Ricklefs 2014; Valkiūnas 2015). Because natural haemosporidian infections are often light and are difficult to identify to species using microscopic examination of blood films, it is essential to develop molecular markers to aid in diagnosis of natural malarial and other haemosporidian infections (Dimitrov et al. 2014). Additionally, such markers are essential for parasite species diagnostic both on the tissue stage in avian hosts and the sporogony stage in vectors (Valkiūnas et al. 2014; Clark et al. 2015).

We used five PCR essays, which target both long (approximately 1000 bp) and short (approximately 350 bp) DNA fragments and are broadly used in diagnostic and phylogenetic research of avian haemosporidian parasites in wildlife (Table 2). Applying more than one PCR protocol, targeting shorter fragments and more conserved gene regions often increase the sensitivity of haemosporidian detection (Valkiūnas et al. 2008; Perkins 2014), but that was not a case with *H. ciconiae* in this study. It is worth noting that short DNA fragments (<300 bp) usually are of low value in haemosporidian phylogenetic research and molecular characterisation of parasites (Fallon et al. 2003; Dinhopl et al. 2015); they can be recommended to use mainly for estimation prevalence of haemosporidians belonging to certain genera. Additionally, for reliable diagnostic of parasites, it is important to optimize PCR protocols because they may not work similarly well across laboratories depending on DNA extraction methods, taq-DNA and other reagent supplier, PCR machine, etc. (Freed and Cann 2006). All applied PCR protocols (Table 2) were optimised and worked well in our laboratory (Bernotienė et al. 2016). It is often deemed that PCR-based diagnostic is a more sensitive tool in determining light infections of haemosporidian parasites (Jarvi et al. 2002; Richard et al. 2002; Durrant et al. 2006; Clark et al. 2014). However, all used PCR assays showed false negative results in this study: gametocytes of *H. ciconiae* were visible by microscopic examination in all blood films using an established microscopy protocol (Valkiūnas et al. 2008), but PCR amplification of the parasite DNA was negative. There might be following explanations of this observation.

Firstly, it is probable that light *Haemoproteus* spp. parasitemia (of ≤0.001 %) might be often undetectable by the PCR amplification due to insufficient starting material of parasite DNA in comparison to huge amounts of bird DNA in each blood sample (Hellgren et al. 2004). This conclusion is in accord with observations by Jarvi et al. (2002), who showed that the application of serological diagnostic methods provided better results in detection of avian malaria infections in comparison to PCR. It is worth mentioning, however, that microscopic examination of blood films was of poor diagnostic sensitivity in the study by Jarvi et al. (2002) probably because these authors examined only ~50,000 red blood cells for parasite detection, and that is not enough for determining haemosporidian parasite-positive samples if parasitemia is ≤0.001 %, which is a common case in wildlife (Valkiūnas 2005).

Secondly, primer sets, which were used in the PCR assays (Table 2), have been originally developed using DNA sequences of *Plasmodium* or *Haemoproteus* parasites inhabiting passeriform birds. These primers might be insufficiently sensitive in amplification of DNA of some distantly related haemoproteids developing in non-passerine birds.

Thirdly, several recent studies reported lack of amplification of haemosporidian parasite DNA from blood samples, in which blood stages were readily visible and even present at high parasitemia, as confirmed by the microscopic examination of blood film (Zehtindjiev et al. 2012; Schaer et al. 2015). It was speculated that some haemosporidians might lose their cyt *b* gene. This hypothesis is unexpected given our present

knowledge about unique haemosporidian parasite morphology and their mitochondrial DNA organization (Wilson and Williamson 1997; Valkiūnas 2005), but it is worth the attention of researchers from an evolutionary perspective. However, even if this hypothesis is correct in some haemosporidian parasites, the DNA of new parasite should have amplified using apicoplast gene primers (Table 2). Because that was not the case in this study, we believe that the first and the second explanations are more likely the case.

This study provides new data indicating that a combination of advanced molecular and microscopic approaches remains essential for reliable comparative research of haemosporidian parasites, particularly during parasite biodiversity investigations (Dimitrov et al. 2014; Clark et al. 2015).

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