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New *Haemoproteus* parasite of parrots, with remarks on the virulence of haemoproteids in naive avian hosts

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ABSTRACT

Haemoproteus infections can cause fatal disease in parrots (Psittaciformes), one of the most endangered groups of birds. The great diversity of parrots in tropical and subtropical ecosystems has been markedly understudied in terms of their parasite diversity. Only two psittacine *Haemoproteus* species have been described. Here we report a new *Haemoproteus* parasite, *H. (Parahaemoproteus) homohandai* n. sp. (lineage hARCHL01) found in erythrocytes of a Red-and-green macaw *Ara chloropterus*. We morphologically and genetically characterize the parasite based on a segment of the mitochondrial cytochrome *b* gene, which can be used for identification and diagnosis of infection. This is the first *Haemoproteus* species described from South American parrots and the first genetically characterized psittacine *Haemoproteus* sp. *Haemoproteus homohandai* n. sp. can be readily distinguished from other haemoproteids by its growing circumnuclear and close to circumnuclear macrogametocytes, which are strictly associated with erythrocyte nuclei, but do not touch the erythrocyte envelope along their entire margin and do not fill erythrocytes up to their poles. Illustrations of growing and mature gametocytes of the new species are given, and a phylogenetic analysis identifies the position of this parasite lineage in relation to other *Haemoproteus* parasites. Importantly, *H. homohandai* n. sp. and all other *Haemoproteus* lineages reported from parrots cluster with species of the subgenus *Parahaemoproteus*, indicating the transmission by *Culicoides* biting midges.

1. Introduction

Blood parasites of the order Haemosporida are cosmopolitan in birds, with the greatest diversity reported from countries with warm climates (Atkinson et al., 2008; Clark et al., 2014). Several haemosporidian species can cause severe disease in domestic chicken, ducks, geese, turkeys, pigeons and domesticated ostriches (Valkiūnas, 2005). Wild birds also succumb to infection due to various haemosporidian infections (Karstad, 1965; Stone et al., 1971; Atkinson and Forrester, 1987; Gabaldon and Ulloa, 1980; Atkinson et al., 2008; Howe et al., 2012; Chagas et al., 2017). During the past 15 years, numerous avian haemosporidian parasites have been characterized using DNA (see MalAvi database, http://mbio-serv2.mbioekol.lu.se/Malavi), providing the opportunity to detect infections in avian hosts not only during intraerythrocytic development, but also in internal organs, which can be markedly damaged by exo-erythrocytic stages (Valkiūnas and Iezhova, 2017). The combination of molecular diagnostic tools and histopathology in recent studies suggests that widespread haemosporidian parasites belonging to the genus *Haemoproteus*, which are relatively benign in adapted natural hosts (Bennett et al., 1993), can cause severe and lethal disease in naive bird species (Donovan et al., 2008; Olias et al., 2013; Valkiūnas and Iezhova 2017). The negative impact of *Haemoproteus* infections on unadapted wild bird populations including parrots remains insufficiently understood (Ilgūnas et al., 2016; Moens et al., 2016). In contrast, it is well-documented that several species of captive parrots in Europe can become infected and die from infection with *Haemoproteus minutus* (see Olias et al., 2011; Palinauskas et al., 2013). This parasite is widespread and prevalent in several species of thrushes (Turdidae) in the Holarctic, and likely the same disease is broadly distributed in captive parrots. There is a risk of introduction into tropical ecosystems by either migrating birds or translocation through commercial trade of infected.

Parrots (Psittaciformes) are among the most endangered bird groups, with about 30% of species being endangered (ICUN Red List 2016, www.iucnredlist.org). Unidentified *Haemoproteus* infections have been reported in parrots globally in tropical and subtropical countries (Bennett et al., 1982; Bishop and Bennett, 1992), but both morphospecies and genetic diversity of these parasites remain insufficiently

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studied. Only two *Haemoproteus* species have been morphologically described from parrots so far which most likely markedly underestimates the diversity of psittacine *Haemoproteus* spp. Molecular markers for diagnostics of parrot *Haemoproteus* morphospecies are absent. Here, we describe a new species of *Haemoproteus* from the Red-and-green macaw *Ara chloropterus,* provide markers for molecular detection of this infection and discuss taxonomy, phylogenetic relationships, prevalence, virulence and possible vectors of haemoproteids of parrots.

2. Material and methods

2.1. Collection of blood samples

Blood films prepared from one adult Red-and-green macaw *Ara chloropterus* of unknown age and gender were examined by microscopic examination of blood films by H. Pendl in 2002. According to the referring veterinarian, the bird was wild caught, imported from Surinam, and had been kept as a pet together with another macaw by a private owner in Austria for several years. This individual bird was still alive in 2017. Routine microscopic examination revealed the presence of haemosporidian parasites, and blood films were submitted for further differentiation to G. Valkiūnas and P. Olias.

Blood films were air-dried within several seconds after their preparation, fixed in methanol and stained with Wright-Giemsa. Blood films were examined for 10–15 min at low magnification (×400) and then at least 100 fields were studied at high magnification (×1000). Intensity of infection was estimated as a percentage by actual counting of the number of parasites per 1000 red blood cells. To determine possible presence of co-infections with other haemosporidian parasites, the entire hapantotype blood film was examined microscopically at low magnification.

2.2. Morphological analysis

An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) was used to examine slides, to prepare illustrations, and to take measurements. The morphometric features studied (Table 1) are those defined by Valkiūnas (2005). Student's *t*-test for independent samples was used to determine statistical significance between mean linear parameters. A *P*-value of 0.05, or less, was considered significant.

2.3. DNA extraction, PCR amplification, and sequencing

The coverslip of one blood smear was removed with xylol. After rinsing with 100%, 95% and 70% EtOH, the smear was air dried and 80 µl of T1 lysis buffer (NucleoSpin Tissue XS, Macherey Nagel, Dürren, Germany) was added to remove the blood cells. Eluted blood cells were incubated together with Proteinase K at 56 °C for 1 h and subsequently processed as described in the manufacturer's instruction. DNA was amplified by nested-PCR reaction using GoTaq polymerase (Promega, Madison, WI) and primers HaemNFI (fw: 5′-CATATATTAAGAGAAITATGGAG-3′) and HaemNR3 (rev: 5′-ATAGAAAGATAAGAAATACCATTC-3′) for the first reaction and HaemF (fw: 5′-ATGGTGCTTTCGATATATGCATG-3′) and HaemR2 (rev: 5′-GCATTATCTGGATGTGATAATGGT-3′) for the nested reaction (Hellgren et al., 2004). The initial PCR conditions were 94 °C for 3 min and 20 cycles of 30 s at 94 °C, 30 s at 50 °C, 45 s at 72 °C with a final step of 10 min at 72 °C. The subsequent nested reaction was run with 35 cycles using 1 µl of the amplicon of the first reaction as template. The amplicon was send for sequencing with primers HaemF and HaemR2 (Seqlab, Göttigen, Germany).

Table 1
Morphometry of host cells and mature gametocytes of *Haemoproteus homohandai* n.sp. from the Red-and-green macaw *Ara chloropterus*.

Feature	Measurements (µm)[a]
Uninfected erythrocyte	
Length	12.5–15.5 (14.0 ± 0.9)
Width	6.6–7.6 (7.1 ± 0.3)
Area	70.3–96.1 (79.3 ± 6.9)
Uninfected erythrocyte nucleus	
Length	5.3–7.0 (6.0 ± 0.5)
Width	1.7–2.6 (2.2 ± 0.3)
Area	9.0–14.5 (10.9 ± 1.2)
Macrogametocyte	
Infected erythrocyte	
Length	13.9–17.0 (15.5 ± 0.8)
Width	6.8–8.4 (7.6 ± 0.4)
Area	81.3–107.6 (92.8 ± 5.8)
Infected erythrocyte nucleus	
Length	4.7–6.6 (5.6 ± 0.6)
Width	2.1–2.7 (2.4 ± 0.2)
Area	9.2–12.8 (10.9 ± 0.8)
Gametocyte	
Length	21.4–30.0 (24.9 ± 2.3)
Width	1.6–2.6 (2.1 ± 0.3)
Area	45.1–66.1 (54.3 ± 5.5)
Gametocyte nucleus	
Length	2.0–4.1 (2.6 ± 0.6)
Width	1.2–2.5 (1.8 ± 0.3)
Area	2.4–6.8 (4.0 ± 1.0)
Pigment granules	14.0–28.0 (22.2 ± 3.5)
NDR[b]	0.9–1.1 (1.0 ± 0.1)
Microgametocyte	
Infected erythrocyte	
Length	13.7–16.5 (15.4 ± 0.8)
Width	7.2–8.5 (7.6 ± 0.3)
Area	83.3–104.7 (93.9 ± 5.8)
Infected erythrocyte nucleus	
Length	4.1–6.9 (5.7 ± 0.6)
Width	1.7–2.7 (2.2 ± 0.2)
Area	9.5–12.1 (10.6 ± 0.8)
Gametocyte	
Length	18.7–26.7 (23.4 ± 2.7)
Width	1.9–3.1 (2.6 ± 0.3)
Area	38.2–72.1 (54.7 ± 10.4)
Gametocyte nucleus[c]	
Length	–
Width	–
Area	–
Pigment granules	17.0–25.0 (20.6 ± 2.6)
NDR	0.7–1.0 (0.9 ± 0.1)

[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 ratio according to Bennett and Campbell (1972).
[c] Morphometry of microgametocyte nuclei is not given because of their markedly diffuse structure, unclear outline and resulting difficulties to measure.

2.4. Phylogenetic analysis

We used the software MEGA7 (version 7.0.21) (Kumar et al., 2016) to investigate the relationship of the mitochondrial cytochrome *b* (*cytb*) sequence obtained from the blood smear of the Red-and-green macaw and other *cytb* sequences published from parrots infected with haemoproteids of subgenera *Haemoproteus* and *Parahaemoproteus*. For comparison, sequences were trimmed and aligned with CLUSTALW. For phylogenetic reconstruction the maximum likelihood (ML) method was used under the GTR substitution model and gamma distributed rates among sites (number of substitution rate categories = 4). The tree with the highest log likelihood (−1885.8990) is shown (Fig. 2). In addition, the maximum parsimony (MP) method was used, and the most parsimonious tree with length = 315 is shown (Fig. 2). The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) are shown next to branches