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Author(s)	Salakij, Jarernsak; Lertwatcharasarakul, Preeda; Kasorndorkbua, Chaiyan et al.
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NOTE

Plasmodium circumflexum in a Shikra (*Accipiter badius*): Phylogeny and ultra-structure of the haematozoa

Jarernsak Salakij[a], Preeda Lertwatcharasarakul[b], Chaiyan Kasorndorkbua[c] and Chaleow Salakij[b, *]

[a]Department of Large Animal and Wildlife Clinical Sciences, Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen, Nakorn Pathom, 73140, Thailand

[b]Department of Pathology, Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen, Nakorn Pathom, 73140, Thailand

[c]Department of Pathology, Faculty of Veterinary Medicine, Kasetsart University, Bangkhen, Bangkok, 10900, Thailand

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Abstract

A wild-caught, juvenile Shikra (*Accipiter badius*) was evaluated for rehabilitation at the Kasetsart University Raptor Rehabilitation Unit (KURRU) with a history of weakness. *Plasmodium* sp. was observed by both light and electron microscopy in blood obtained on day 1 of evaluation. Based on the appearance of erythrocytic meronts and gametocytes, the parasites were defined as *Plasmodium* (*Giovannolaia*) *circumflexum*. The sequence analysis of the mitochondrial cytochrome *b* gene from the plasmodia was closely related to parasites found in the Grey-headed woodpecker from Myanmar and the Brown hawk-owl from Singapore. Transmission electron microscopic examination revealed organelles in the haematozoa and heterophils that ingested the plasmodia. This is the first recorded case of *Plasmodium circumflexum* in a wild Shikra. This note emphasises the molecular characterisation and ultra-structure of the haematozoa.

Key words: Avian malaria, Cytochrome *b* gene, Raptor

Avian malaria is a common mosquito-transmitted disease of wild birds that is caused by a group of *Plasmodium* species of the phylum Apicomplexa. These parasites can be pathogenic to penguins, domestic poultry, ducks, raptors, pigeons, waterfowl and the passerines in Hawaii[1]. Despite that, many infected birds do not become ill and are commonly asymptomatic carriers[8]. In a review of papers examining avian blood parasites, Bennett *et al*.[3] found that only 4% reported pathogenicity in birds, with most pathogenic cases dealing with domestic birds or zoological birds. The Shikra (*Accipiter badius*, Gmelin, 1788) is a diurnal bird of prey in the

*Corresponding author: Chaleow Salakij, Department of Pathology, Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen, Nakorn Pathom, 73140, Thailand
Phone: +66-34-351-901. Fax: +66-34-351-405. E-mail: fvetcls@ku.ac.th

Family Accipitridae and commonly resides in Thailand[5]. The aim of this note was to determine the ultra-structure and phylogenetic relationships of the *Plasmodium* found in a Shikra.

One female Shikra with juvenile plumage, weight 0.25 kg, was submitted to the Kasetsart University Raptor Rehabilitation Unit (KURRU) with a history of weakness on August 2, 2010. The bird was caught in Bangkok then submitted to the unit. The bird was estimated to be approximately five months-old, as the bird was fledged in May of the same year. Upon clinical examination, the bird was alert and the pectoral muscle was adequate. There were no abnormalities in any part of the body.

Four hundred microliters of blood (EDTA-anticoagulated) were collected from the jugular vein. Blood smears were prepared, air-dried and stained with Wright's stain (Merck KGaA, Darmstadt, Germany) for grading of the blood parasite infection, hypochromasia and polychromasia via actual counting of the number of parasites per 10,000 erythrocytes (RBC). EDTA samples were processed for electron microscopy as described previously[11]. The light microscopic and ultra-structural characteristics of haematozoa were then evaluated.

The erythrocytic stages of *Plasmodium* sp. were detected in the blood smear and some RBC were hypochromic (Figs. 1A, 1D). The percentage of RBC parasitised by *Plasmodium* sp. was 12.9%. Trophozoites (Figs. 1D, 1H) were located polar or subpolar to the RBC nuclei. Gametocytes were elongated in form and had a position lateral to the nuclei of the infected RBC (Fig. 1D). Erythrocytic meronts were larger than the nuclei of infected RBC (Fig. 1A). Intra-cytoplasmic pigment granules were brown in colour and presented in all erythrocytic stages (Fig. 1). These erythrocytic stages corresponded to the morphology of *Plasmodium circumflexum* (*P. circumflexum*) subgenus *Giovannolaia* in having elongate gametocytes, and the parasites did not displace the host cell nucleus[1]. Ultra-structurally, the meronts contained 6–12 merozoites (Figs. 1B, 1C, 1G). The merozoites contained nuclei, pigment granules (Fig. 1C) and an apical complex with tear-shaped rhoptries (Figs. 1C, 1G). The gametocytes contained numerous ribosomes and pigment granules (Figs. 1E, 1F).

DNA was extracted from EDTA blood samples following a genomic DNA extraction mini-kit protocol (Favorprep, Taiwan). Cytochrome *b* (cyt *b*) of the mitochondrial DNA of the *Plasmodium* was amplified by nested polymerase chain reaction (PCR) using specific primers designed in a previous report and designated as the HaemNF and HaemNR2 primers for the primarily PCR of amplication[4]. The PCR amplification was performed using recombinant *Taq* DNA polymerase (Invitrogen, USA). The temperature conditions employed for the PCR amplification were as follows: one cycle of denaturation at 94°C/5 min followed by 35 cycles of denaturation at 94°C/30 sec, primer annealing at 50°C/30 sec and primer extension at 72°C/45 sec. The final extension was at 72°C/5 min. The PCR product of the primarily PCR was used as the template for the nested PCR using the HaemF and HaemR2 primers, and the temperature conditions were the same as those used in the primarily PCR. The PCR product was subjected to 1.5% agarose gel electrophoresis. The gel was later stained with ethidium bromide and visualised using a UV transilluminator. The PCR products (478 base pairs) were purified using NucleoSpin® ExtractII (MACHERE Y-NAGEL, Germany) and confirmed by nucleotide sequencing at BioDesign Co., Ltd. (Pathumthani, Thailand). The nucleotide sequences were analysed using BLAST and BioEdit version 7.0.9.0. The data were then used to generate phylogenetic trees with the available submitted database from GenBank using the neighbour-joining method with 1000 replicates implemented in MEGA4 program[12].

The phylogenetic tree (Fig. 2) revealed that the mitochondrial cyt *b* isolate from the *Plasmodium* found in the Shikra (JN639001) was significantly different from that of other reported avian *Plasmodium* but was closely related to the parasite found in the Grey-headed woodpecker

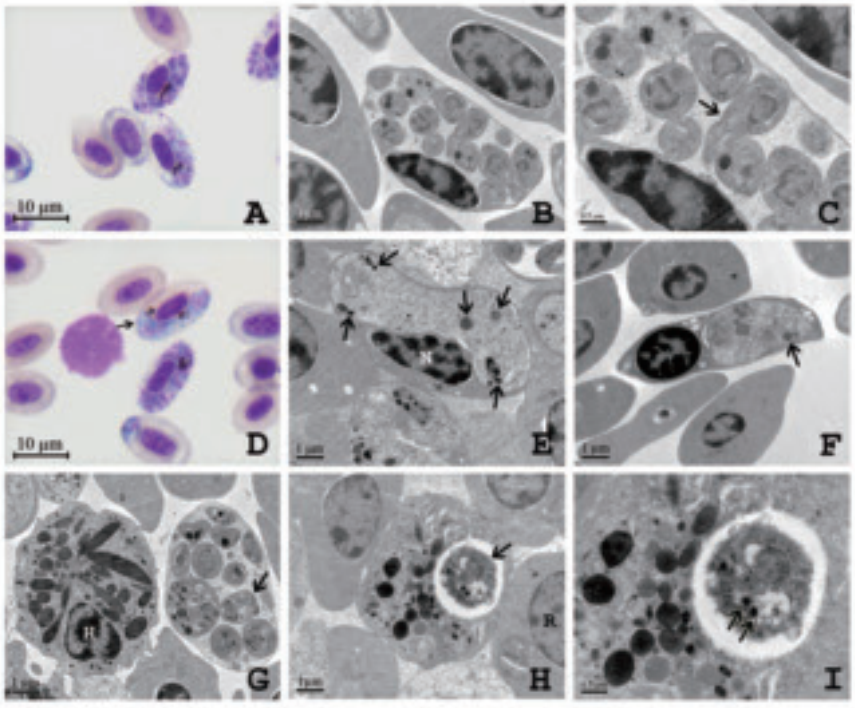

Fig. 1. Light (A, D) and transmission electron micrographs (TEM) of *Plasmodium circumflexum* in a Shikra. A. Two erythrocytes bearing meronts of *P. circumflexum*, Wright's stain. Note the hypochromic RBC (middle cell). B. A mature meront in an erythrocyte containing 12 merozoites. C. Higher magnification image of panel B with one merozoint contained a pair of rhoptries (arrow). D. Three erythrocytes bearing *P. circumflexum*. One erythrocyte contains a gametocyte (arrow). A mature trophozoite in the lower RBC, Wright's stain. E. Longitudinal TEM section of gametocyte. Note the malarial pigments (arrows) and the RBC nucleus (N). F. A cross-section of the gametocyte in RBC. Note the malarial pigments (arrow). G. Heterophil (H) next to a mature *Plasmodium* meront in a RBC. One merozoint contained tear-shaped rhoptry (arrow). H. A heterophil containing a phagocytosed *Plasmodium* (arrow) next to a single trophozoite of a *Plasmodium* in a RBC (R). I. Higher magnification image of a heterophil in panel H with two pigment granules (arrows) in the food vacuole of the plasmodia visible. TEM, Uranyl acetate and lead citrate stains.

(*Picus canus*) from Myanmar (98.9% identity, EF380111) and the Brown hawk-owl (*Ninox scululata*) from Singapore (97.8% identity, AY099035), in addition to *P. circumflexum* strain TURDUS1 found in *Sylvia atricapilla* (96.7% identity, JN164734).

The blood samples obtained on August 16, 2010 (Day 14), 7 months (March 4, 2011) and 1 year (August 21, 2011) later, had no detectable *Plasmodium* sp. The percentage of polychromatophilic RBC on Day 14, 7 months and 1 year later were 1.5%, 6.7% and 1.2% respectively. The bird was still in the rehab unit without any treatment and remained clinically healthy at the time of writing.

This case is the first report of *P. circumflexum* in the blood of a Shikra from Asia and to characterise the parasite at the molecular level. *P. circumflexum* has a cosmopolitan distribution in passerines and is also found in grouse, geese and waders. It is mildly pathogenic in its natural hosts[6]. Hypochromic RBC (5.1%) and polychromatophilic RBC (13.9%) reflect severe malarial infections and regenerative response from the host[8]. However, the Shikra presented no other clinical signs of anaemia except weakness at presentation, and *Plasmodium* was not detected in blood smears as little as 14 days later. The malarial parasitemia on Day 1 might be due to the weakness of the Shikra. The disappearance of malarial-infected RBC on Day 14, without any treatment related to the hemoparasitism affirm the mild pathogenicity of *P. cirmcumlexum* in the Shikra.

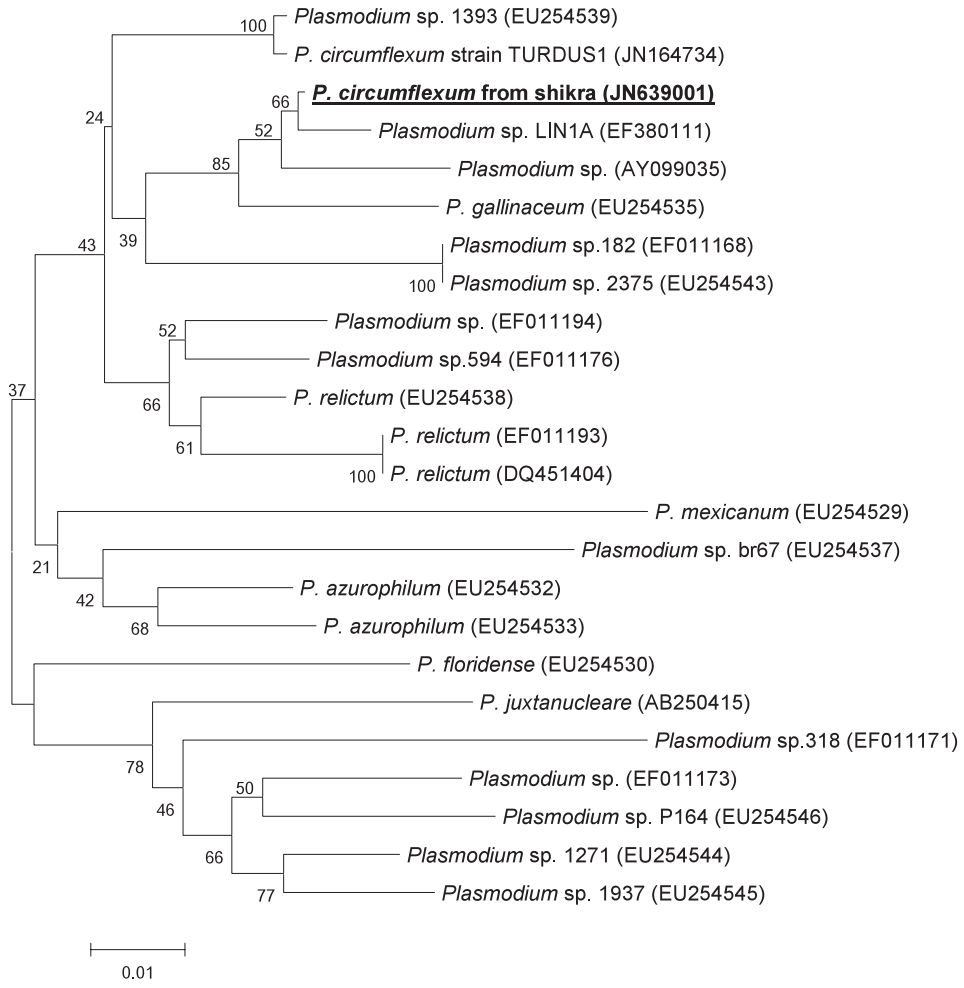

Fig. 2. Phylogenetic tree based on the partial cytochrome *b* gene of mitochondrial DNA from the *Plasmodium circumflexum* isolated from a Shikra (*Accipiter badius*) in Thailand (JN639001). Other sequences were obtained from GenBank. Genbank Accession numbers are shown in parentheses. Bootstrap values obtained from 1000 replications are shown at branch. The scale bar represents the number of substitutions for a unit branch length.

Plasmodia have been previously documented in some raptors and in migrating accipitrine hawks[10]. There have been 11 species of *Plasmodium* found in birds of prey[2,13]. The species associated with mortality include *P. relictum, P. elongatum, P. circumflexum* and *P. cathemerium*[10]. *P. circumflexum* was reported in 40.6% (n = 96) of sampled sharp-shinned hawk (*Accipiter striatus*) from Southern New Jersey, as assessed by blood culture[9]. The *Plasmodium* species found in the sharp-shinned hawk from Vermont, USA (EU254539) are not closely related to those found in the Shikra (Fig. 2). There has been a high prevalence (34.0% of 699 birds) of avian malaria detected in previous studies in Asia, and based on sequence data of the cyt *b* gene, there are 34 distinct lineages of *Plasmodium* in Myanmar, India and South Korea[7]. Anyhow, the Shikra was not included in their study.

The evidence of degenerative malaria in the vacuoles of heterophils observed by electron microscopy supported that these cells maybe play a major role in eliminating the plasmodia. This note, with an emphasis on the phylogeny and ultra-structure of the haematozoa, presents the first report of a wild Shikra naturally infected with avian malaria.

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