Molecular characterization of Polychromophilus parasites of Scotophilus kuhlii bats in Thailand

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Abstract

Parasites of the haemosporidian genus Polychromophilus have exclusively been described in bats. These parasites belong to the diverse group of malaria parasites, and Polychromophilus presents the only haemosporidian taxon that infects mammalian hosts in tropical as well as in temperate climate zones. This study provides the first information of Polychromophilus parasites in the lesser Asiatic yellow bat (Scotophilus kuhlii) in Thailand, a common vespertilionid bat species distributed in South and Southeast Asia. The gametocyte blood stages of the parasites could not be assigned to a described morphospecies and molecular analysis revealed that these parasites might represent a distinct Polychromophilus species. In contrast to Plasmodium species, Polychromophilus parasites do not multiply in red blood cells and, thus, do not cause the clinical symptoms of malaria. Parasitological and molecular investigation of haemosporidian parasites of wildlife, such as the neglected genus Polychromophilus, will contribute to a better understanding of the evolution of malaria parasites.

Introduction

Malaria parasites (order Haemosporida) infect birds, squamates, chelonians and several groups of mammals, including humans, and are transmitted by different groups of haematophagous dipterans (Garnham, 1966). The human-infecting parasite species belong to the genus Plasmodium, which is only one out of at least 15 genera that together compose over 500 haemosporidian species. Parasites of this diverse group differ in host specificities, adaptations and their life cycles (Garnham, 1966). For instance, all haemosporidian genera, except Plasmodium, lack the distinct replication phase inside red blood cells, which is the exclusive cause of clinical symptoms of malaria. Therefore, studying the diversity and evolution of the entire haemosporidian parasite group will contribute to our understanding of the important malaria disease in humans (Galen et al., 2018).

Parasites of the haemosporidian genus Polychromophilus are transmitted by ectoparasitic highly specialized nycteribiid flies and have exclusively been described in bats (Dionisi, 1898; Garnham, 1966, 1973; Witsenburg et al., 2012). Polychromophilus presents the only haemosporidian taxon that infects mammalian hosts in tropical as well as in temperate climate zones. These parasites are common in bats in Europe and in the tropical regions of Africa, Asia, Australia and South America (e.g. Garnham, 1966; Perkins and Schaer, 2016). Even though Polychromophilus parasites are widespread and common, only five morphospecies have been formally described to date. Polychromophilus murinus has been mainly reported in bats of the family Vespertilionidae and Polychromophilus melanipherus in bats of the family Miniopteridae (e.g. Garnham, 1966; Gardner and Molyneux, 1988). The species Polychromophilus corradetti and Polychromophilus adami have been described from African Miniopterus species (Landau et al., 1980). The description of Polychromophilus deanei from Myotis nigricans (Vespertilionidae) in Brazil, and three other records of Polychromophilus from bats in Brazil and Southern USA provided evidence of chiropteran haemosporidian parasites in the New World (Wood, 1952; Deane and Deane, 1961; Garnham et al., 1971; Foster, 1979). Several phylogenetic studies have confirmed that P. murinus and P. melanipherus comprise distinct species (e.g. Megali et al., 2011; Witsenburg et al., 2012), the latter possibly representing a species complex (Duval et al., 2012). In molecular phylogenies, sequences from Polychromophilus of M. nigricans from Panama, which might represent P. deanei, group closely with P. murinus parasite sequences (Borner et al., 2016). The remaining two morphospecies have not been included in phylogenetic analyses yet, however Polychromophilus sequences sampled from the African Miniopterus host species of P. corradetti and P. adami grouped within the P. melanipherus clade (Duval et al., 2012; Rosskopf et al., 2019).

Very few studies have focused on morphological or molecular investigations of Polychromophilus parasites in Asia. Two morphological studies described Polychromophilus from hipposiderid bat species in Thailand and Malaysia (Eyles et al., 1962; Landau et al., 1984). One molecular study published a Polychromophilus sequence from the vespertilionid bat Kerivoula hardwickei in Cambodia and a recent study published two short cytochrome
b sequences for *P. murinus* and *P. melanipherus* from *Myotis siligorensis* (Vespertilionidae) and *Taphozous melanopogon* (Emballonuridae) in Thailand (Duval et al., 2007; Arnuphapprasert et al., 2020). Here, data are presented from molecular investigations of *Polychromophilus* infections in the lesser Asiatic yellow bat (*Scotophilus kuhlii*) in Thailand that were originally reported as unidentified haemosporidian parasites in a preliminary morphological study on white blood cell counts of *S. kuhlii* (Chumnannde and Pha-Obnga, 2018) and add important information to the phylogeny of these neglected parasites.

**Materials and methods**

Bats were captured in April 2018 in the Muang district in the Nakhon Phanom province in Thailand (17°24′38.92″N and 104°46′42.82″E) using standard mist nets. A total of 44 bats were captured from the same colony. Standard morphological measurements were taken for each bat and the identification keys of Duengkae (2007) and Srinivasulu et al. (2010) were used for species identification. Bats were kept individually in cotton bags. Blood sampling followed approved animal care protocols and comprised 0.6–1.0% body mass of blood (e.g. 6–19 μL g⁻¹) per bat (e.g. Predict One Health Consortium, 2013). The blood samples were used to prepare two thin blood smears and to preserve blood on DNA FTA cards. Bats were released at the capture side, once they had fully recovered. The thin blood smears were fixed and stained with Wright-Giemsa (following Paksuz et al., 2009). Slides were thoroughly scanned by light microscopy with a magnification of ×1000 using oil immersion. The morphology of the blood stages of the parasites was compared to original species descriptions. Parasitaemia was calculated as the percentage of parasite-infected erythrocytes in the total number of erythrocytes (total number of parasites/products of mean number of erythrocytes per field × number of counted fields). The mean number of erythrocytes per field was determined by counting three fields and the number of parasites was recorded in 50 fields (fields with comparable erythrocyte density).

Whole genomic DNA was extracted from blood dots on DNA FTA cards using the DNeasy extraction kit (Qiagen). Two mitochondrial genes of the bats were amplified and sequenced to verify the morphological bat species identifications were confirmed with molecular barcoding. The whole mitochondrial cytochrome *b* was sequenced, which featured a 99.7% nucleotide identity with the *S. kuhlii* reference sequences (e.g. EU750921) in GenBank. In addition, 928 bp of the mitochondrial NADH dehydrogenase subunit 1 were sequenced and nucleotide identity with an *S. kuhlii* reference sequence (AB079818) was 98.9% (accession numbers listed in Table S3).

The blood stages of *Polychromophilus* parasites are limited to gametocytes and the morphology corresponds to the description of *Polychromophilus* parasites of vespertilionid hosts. In Giemsa-stained blood smears, the immature parasites feature a pale cytoplasm and the nucleus is located peripherally and stains purple (Fig. 1A a). When mature, the gametocytes fill the host cell completely and cause a slight enlargement of the erythrocyte. Fine hemozoin pigment grains are scattered in the cytoplasm, a characteristic that is attributed to *P. murinus* (Fig. 1A b–f). In marked contrast, the pigment of *P. melanipherus* is much larger and coarse-grained. The male microgametocytes feature a light pink-stained cytoplasm (Fig. 1A b–c), whereas the female macrogametocytes stain purple-blue (Fig. 1A d–f), both exhibiting a small distinct pink-staining nucleus that is placed eccentrically. The morphology of the gametocyte stages did not allow a clear assignment to any described morphospecies.

The mean *Polychromophilus* gametocytemia in the blood smear-positive samples was 0.05% (minimum of 0.01% and maximum of 0.1%) (Fig. 1B).

Sanger sequencing revealed that the parasite *cytb* nucleotide sequences were identical, while we noted that the *cox1* sequences in one out of five samples differed by one base. Hence, the five *S. kuhlii* individuals were infected with one cytochrome *b* haplotype and two cytochrome oxidase 1 haplotypes of the same *Polychromophilus* species.

The three-genome phylogeny of *Polychromophilus* in the context of the major haemosporidian parasite clades recovered the *Polychromophilus* parasites (Fig. 2, highlighted in orange) as sister clade to a group that contains the lizard and bird *Plasmodium* species (highlighted in yellow), confirming previous studies that showed a distant relationship of *Polychromophilus* parasites to *Plasmodium* and *Hepatocystis* of mammalian hosts (highlighted in grey) (Fig. 2). Together, they group with the *Plasmodium* species of ungulates (Fig. 2, highlighted in blue). All *Polychromophilus* sequences group into one monophyletic clade (posterior probability of 1) that contains two main subclades. The first distinct subclade comprises all sequences of *P. melanipherus* of *Miniopterus* bat hosts (and one parasite sequence of a *Taphozous* bat host) and the second subclade exclusively includes sequences of *Polychromophilus* parasites of vespertilionid (and one rhinolophid) bat species, confirming a clear separation of parasites of miniopterid and vespertilionid hosts. The second subclade contains *P. murinus* sequences from bats in Europe, Madagascar and Thailand and one sequence that is basal to *P. murinus*, a sample from *M. nigriceps* from Panama. The placement of the sample from *K. hardwickii* from Cambodia could not be resolved. The other subclade that is separated from the *‘P. murinus’* clade contains the sequences of *Polychromophilus* of *S. kuhlii* from Thailand (Fig. 2, highlighted in green) and two parasite samples from *Pipistrellus alf. grandisleri* and *Laephotes capensis* from Guinea.

**Results**

The survey of haemosporidian parasites in a colony of *S. kuhlii* bats identified *Polychromophilus* infections in five out of 44 bat individuals (prevalence = 11%). This is the first host record for the vespertilionid bat genus *Scotophilus* and for the species *S. kuhlii* for infections with *Polychromophilus* parasites. The morphological bat species identifications were confirmed with molecular barcoding. The whole mitochondrial cytochrome *b* was sequenced, which featured a 99.7% nucleotide identity with the *S. kuhlii* reference sequences (e.g. EU750921) in GenBank. In addition, 928 bp of the mitochondrial NADH dehydrogenase subunit 1 were sequenced and nucleotide identity with an *S. kuhlii* reference sequence (AB079818) was 98.9% (accession numbers listed in Table S3).

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Fig. 1. (A) Representative Giemsa-stained micrographs of gametocyte blood stages of *Polychromophilus* parasites from *Scotophilus kuhlii* in Thailand (a, c–d from bat sample CC-33; b, f from bat sample CC-28). Size bars = 5 μm, magnification = 1000×. (a) Immature gametocyte with pale cytoplasm and a peripheral purple nucleus. (b–f) Mature gametocytes that entirely occupy and slightly enlarge the host erythrocytes. The malaria pigment hemozoin is visible as fine dark grains scattered throughout the cytoplasm. (b–c) Male microgametocytes with the cytoplasm in a characteristic light pink colour and the small nucleus in a slightly darker pink. (d–f) Female macrogametocytes with a purple-blue cytoplasm and small nuclei in pink. (B) Parasitaemia in %. Parasitaemia values in the five infected *S. kuhlii* ranged between 0.01 and 0.1% (prevalence of 11%, 5/44 *S. kuhlii* infected). Inserted photograph of *S. kuhlii*.

**Discussion**

This study provides the first information on haemosporidian parasites in the bat species *S. kuhlii* in Thailand. The morphology of the blood stages and the phylogenetic analysis identify the parasites as belonging to the genus *Polychromophilus*. The infections featured low overall parasitaemias as reported from other *Polychromophilus* infections (e.g., Rosskopf et al., 2019). The three-genome phylogeny confirms a clear separation of *Polychromophilus* parasites of *Miniopterus* bat species and of vespertilionid bat species, the latter including the parasites of *S. kuhlii*. The phylogenetic analysis recovered the *Polychromophilus* parasites as sister clade to a group that contains the lizard and bird *Plasmodium* species, as shown before (Witsenburg et al., 2012). However, the most comprehensive phylogeny based on multiple nuclear markers clearly placed *Polychromophilus* as sister clade to the unglazed *Plasmodium* species (Galen et al., 2018).

Thus, the placement of *Polychromophilus* as sister to the avian/lizard *Plasmodium* species in our analysis can likely be attributed to the unavailability of *cox1*, *clpC* and *EF2* sequences for the majority of the *Polychromophilus* references that were included in the analysis (Tables S2 and S3). Genes display different rates and patterns of evolution and analysing genes of the parasites’ three genomes for robust phylogenies of haemosporidian parasites has been established (e.g., Martinsen et al., 2008). However, many phylogenetic studies are still limited to the analysis of (rather short) cytchrome *b* sequences.

To date, only four studies have reported *Polychromophilus* parasites from Asian bats. Eyles et al. (1962) reported *Polychromophilus* parasites in the bat species *Hipposideros bicolor* in Malaysia and described the gametocytes as oval in shape, with clear-cut borders and that the parasites only partially occupy the host erythrocytes (Eyles et al., 1962). Another morphological study described *Polychromophilus* from *Hipposideros larvatus* in Thailand (as *Biguettella minuta* which was considered as a vicariant form of *Bioccala*, a subgenus of *Polychromophilus*) (Landau et al., 1984). The gametocytes of the latter were also described as not filling the host cell. Thus, the gametocytes of *Polychromophilus* from *Hipposideros* differ from the morphology of the mature gametocytes observed in the current study that fill the entire host cells and even slightly enlarge the erythrocytes. The only study that reported *Polychromophilus* from a vespertilionid bat species in Asia is that of Duval et al. (2007) that found *K. hardwickii* in Cambodia infected with *Polychromophilus* sp. (Duval et al., 2007). In our phylogenetic analysis, the nucleotide sequence of *Polychromophilus* of *K. hardwickii* is separated from *Polychromophilus* of *S. kuhlii*. Therefore, we assume that the *Polychromophilus* parasites of *S. kuhlii* in Thailand do not represent the parasites detected in Asian hipposiderid hosts nor the *Polychromophilus* parasite reported from *K. hardwickii* in Cambodia. The phylogenetic analyses resulted in the placement of *Polychromophilus* of *S. kuhlii* outside the *P. melaniapherus* and *P. murinus* clades, which also contain the two recently reported *Polychromophilus* parasites from Thailand (Armuthapprasert et al., 2020). The *Polychromophilus* parasites of *S. kuhlii* form a group with the Guinean *Polychromophilus* parasites that have been suggested to represent a distinct species (Schäfer et al., 2013; Rosskopf et al., 2019). Within this group, the *Polychromophilus* parasites of *S. kuhlii* are clearly separated from the Guinean samples (posterior probability = 1) and might therefore also present a distinct species.

Future morphological studies that investigate the tissue stages and molecular studies of additional *Polychromophilus* parasites of Asian bats are needed to reassess this assumption. The host species *S. kuhlii* is widely distributed in South Asia, southern China and Southeast Asia and is found in primary and secondary habitats, both in rural and urban areas and might represent a species complex (Trujillo et al., 2009; Srinivasulu and Srinivasulu, 2019). Systematic sampling of *S. kuhlii* across its distribution range and of other potential vespertilionid bat host species will add important information on the host specificity, the prevalence and nyncteriibid vectors of *Polychromophilus* parasites in Asia.

**Data**

Nucleotide sequence data reported in this paper are available in the GenBank database under accession nos. MT750305-MT750321.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S003118202000222X.

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**Author contributions.** C.C. and J.S. conceived and designed the study. J.S. and O.W. performed phylogenetic analysis. All authors conducted data gathering and wrote the article.

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Conflict of interest. None.

Ethical standards. The Institutional Animal Care and Use Committee of Nakhon Phanom University (project code B1) reviewed and officially approved this survey (date 13.07.2017). Sampling followed approved animal care protocols (e.g. Predict One Health Consortium, 2013). The authors assert that all procedures contributing to this work comply with the ethical standards of the national and institutional guides on the care and use of animals.

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