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Nycteria parasites of Afrotropical insectivorous bats ☆

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ABSTRACT

Parasitic protozoan parasites have evolved many co-evolutionary paths towards stable transmission to their host population. *Plasmodium* spp., the causative agents of malaria, and related haemosporidian parasites are dipteran-borne eukaryotic pathogens that actively invade and use vertebrate erythrocytes for gametogenesis and asexual development, often resulting in substantial morbidity and mortality of the infected hosts. Here, we present results of a survey of insectivorous bats from tropical Africa, including new isolates of species of the haemosporidian genus *Nycteria*. A hallmark of these parasites is their capacity to infect bat species of distinct families of the two evolutionary distant chiropteran suborders. We did detect *Nycteria* parasites in both rhinolophid and nycterid bat hosts in geographically separate areas of Sub-Saharan Africa, however our molecular phylogenetic analyses support the separation of the parasites into two distinct clades corresponding to their host genera, suggestive of ancient co-divergence and low levels of host switching. For one clade of these parasites, cytochrome b genes could not be amplified and cytochrome oxidase I sequences showed unusually high rates of evolution, suggesting that the mitochondrial genome of these parasites may have either been lost or substantially altered. This haemosporidian parasite-mammalian host system also highlights that sequential population expansion in the liver and gametocyte formation is a successful alternative to intermediate erythrocytic replication cycles.

1. Introduction

Haemosporidian parasites (phylum Apicomplexa) infect a wide range of mammals including primates, rodents and bats (Garnham, 1966). Best known are haemosporidian parasites of the genus *Plasmodium*, the causative agent of human malaria. This vector-borne infectious disease causes significant mortality and morbidity every year, particularly in infants across the tropical and subtropical zones. A distinct hallmark of *Plasmodium* parasites is asexual replication in host erythrocytes, called merogony (schizogony). The repeated rupture and invasion of red blood cells is a primary cause of disease in infected individuals. In marked contrast, related haemosporidian genera such as *Haemoproteus* and *Hepatocystis* use other tissues for merogony before they invade erythrocytes and develop into gametocytes. It is assumed that haemosporidian parasites that lack blood schizogony are generally less pathogenic (e.g. Valkiunas, 2005), but there are some exceptions where particular tissue stages can cause damage as well (Atkinson and van Riper, 1991). Understanding the phylogenetic relationships of these less pathogenic relatives of the malarial parasites is important for gaining a better understanding of host-parasite coevolution.

Mammals are hosts to parasites of nine haemosporidian genera in addition to *Plasmodium* (Supplementary Table S1). The genus *Hepatocystis* appears to be a derived clade from mammalian *Plasmodium* parasites (Perkins and Schall, 2002; Schaer et al., 2013). *Hepatocystis* parasites infect a wide range of hosts including primates, bats, ungulates and rodents, whereas parasites of the

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank database under accession Nos. KP053763–KP053768, KP053770–KP053788, KP053790–KP053806, KP053808–KP053812.

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genus *Rayella* have been described only from flying squirrels (Sciuridae, Rodentia). Interestingly, parasites of all other seven haemosporidian genera thus far described have been found exclusively in bats, emphasizing that bats might harbor the most diverse set of haemosporidian parasites within the mammalian clade (Garnham, 1966; Schaer et al., 2013). Historical classification of mammalian haemosporidian parasites was done according to morphological and biological characteristics, resulting in initial misplacement of some chiropteran haemosporidian parasites to the genus *Plasmodium*. However, the observation that certain bat parasites lack schizogony in erythrocytes and, therefore, cannot represent the genus *Plasmodium*, led to a reclassification of several species (Garnham, 1950; Perkins, 2014). The incorporation of molecular data into systematic studies of the family also has challenged some previous classifications. For instance, *Hepatocystis* parasites from both bats and primates fall within the clade of mammal-infecting *Plasmodium* spp. in almost all analyses (Perkins and Schall, 2002; Olival et al., 2007; Martinsen et al., 2008; Outlaw and Ricklefs, 2011; Schaer et al., 2013). The placement of the haemosporidian genus *Polychromophilus*, comprising species that infect vespertilionid and miniopterid bat hosts, has been more uncertain. Megali et al. (2010), who produced the first molecular sequences from *Polychromophilus* spp., showed it as an unresolved clade sister to the two non-mammalian *Plasmodium* spp. that they included. Outlaw and Ricklefs (2011) recovered this genus as sister to avian and saurian *Plasmodium* spp., suggesting that invasion of mammals occurred more than once in the evolutionary history of the family. However, the topologies presented in Schaer et al. (2013) instead place the genus *Polychromophilus* as the most basal lineage of an exclusively mammal-infecting parasite clade. This latter analysis included three isolates of yet another bat-specific haemosporidian genus, *Nycteria*. Two of these isolates were from West African *Rhinolophus* spp. and the third was sampled from *Megaderma spasma* in Cambodia (Duval et al., 2007). Here, we sought to expand the study of *Nycteria* parasites and test further their placement in the haemosporidian parasite phylogeny.

The genus *Nycteria* was named after the first reported bat host genus, *Nycteris* (Garnham and Heisch, 1953). Although documented only infrequently, these parasites appear to be prevalent in many parts of the Old World tropics (Fig. 1A, Supplementary Table S2). Early parasite descriptions found in the bat species *Nycteris thebaica* from South Africa by Bowhill (1906), in *Nycteris hispida* from the Democratic Republic of the Congo (DRC) by Rodhain (1926), and in *Nycteris grandis* from Liberia (Theiler, 1930) were retroactively classified into the parasite genus *Nycteria*. This occurred concurrently with a detailed description of the type species, *Nycteria medusiformis*, isolated from *Nycteris* bat species from Kenya and Zanzibar, and a report of similar parasites from *Nycteris capensis* in Sudan (Garnham and Heisch, 1953). Presence of the type species in *Nycteris* hosts was subsequently confirmed (Lips and Rodhain, 1956). Additional host genera and families of *Nycteria* parasites were first recognised when haemosporidian parasites in the bat species *Rhinolophus hildebrandtii* and *Hipposideros caffer centralis* (=*Hipposideros ruber;* Lawrence, 1964) were analysed (Krampitz and de Faveaux, 1960). Despite an initial misclassification as belonging to parasites of the genus *Polychromophilus*, which only occur in vespertilionid and miniopterid bat hosts (Garnham, 1973; Landau et al., 1980), these parasites were later reclassified as *Nycteria congolensis* (Garnham, 1966). Subsequent reports from Gabon, Republic of Congo and Thailand expanded the known *Nycteria* spp. from rhinolophid and nycterid bats and *Hipposideros larvatus*, respectively (Rosin et al., 1978; Landau et al., 1984; Fig. 1A, Supplementary Table S2). The presence of *Nycteria* parasites in *M. spasma* in Cambodia (Duval et al., 2007) as well as another report of *N. medusiformis* from the emballonurid bat, *Taphozous perforatus,* in Egypt (Morsy et al., 1987) indicate that more bat genera and families (Fig. 1B), and from a much wider geographic spread, than currently anticipated can harbor *Nycteria* spp. (Supplementary Table S1).

To date, the characterisation of *Nycteria* spp. or lineages has been based on the morphological description of parasite blood and tissue stages. Similar to the haemosporidian genera *Hepatocystis* and *Polychromophilus, Nycteria* parasite blood infections are limited to gametocytes. Male gametocytes are round and closely resemble *Plasmodium malariae* parasites (Landau et al., 1976). They rarely occupy the host erythrocyte completely, display a distinct limit between nucleus and cytoplasm, frequently exhibit an accessory chromatin dot and few, coarse pigment granules (Landau et al., 2012). An observation of two distinct gametocyte morphologies led to the proposal of two distinct taxonomic

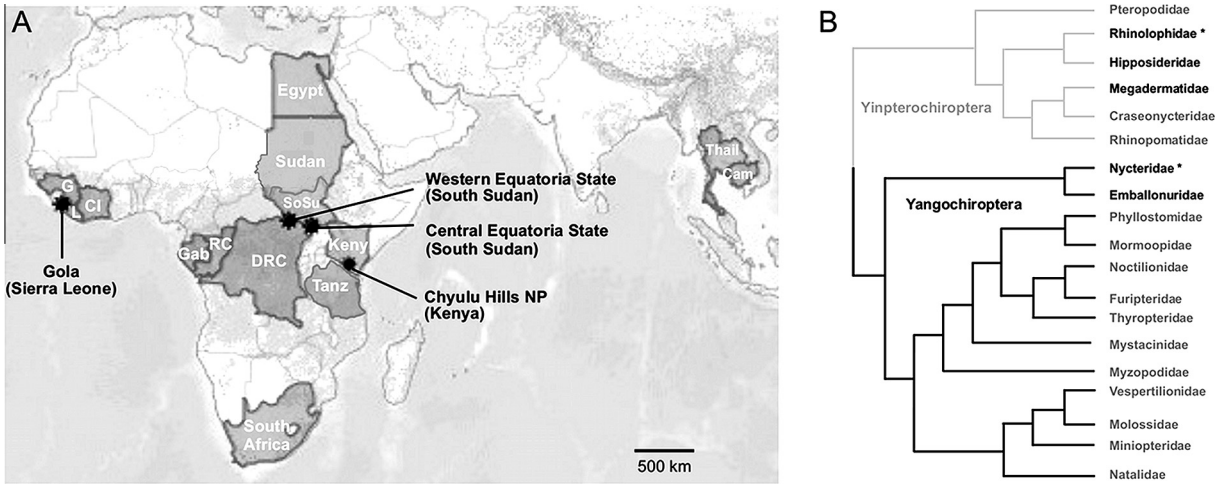

Fig. 1. Locations of bats with documented *Nycteria* parasite infections and Chiroptera phylogeny. (A) Countries with previous records (see also Supplementary Table S2) are highlighted in grey. Locations of *Nycteria* parasite infections from this study are indicated with stars. Cam = Cambodia, CI = Côte d'Ivoire, DRC = Democratic Republic of the Congo, Gab = Gabon, G = Guinea, Keny = Kenya, L = Liberia, RC = Republic of the Congo, SoSu = Republic of South Sudan, Thail = Thailand, Tanz = Tanzania. (B) Schematic overview of chiropteran phylogeny (adapted from Jones and Teeling, 2006). The bat families that were investigated in the current study are indicated in black and those found infected with *Nycteria* parasites are labeled with stars.

groups (Rosin et al., 1978). According to this classification, parasites infecting bats of the genus *Nycteris* produce well-defined gametocytes, whereas those isolated from infected bats of the genus *Rhinolophus* appear more diffuse. The latter implies that the cellular structures of the gametocytes might be less dense and therefore appear paler after Giemsa staining, which is particularly apparent in the nuclei. Asexual replication and parasite population expansion occurs exclusively in the parenchyma cells of the liver, where they develop into peculiar lobulated schizonts (meronts) of less than 100 μm in size, resembling *Plasmodium falciparum* liver stages (Garnham, 1966; Supplementary Fig. S1).

In this study we present, to our knowledge, the first detailed molecular phylogeny of the haemosporidian genus *Nycteria*. By screening candidate bat species captured during biodiversity surveys in eastern and western Africa, we identified two distinct *Nycteria* parasite/bat assemblages.

2. Materials and methods

2.1. Field sampling

2.1.1. Sampling sites

Samples from candidate bat taxa were investigated from South Sudan, Sierra Leone, Kenya and Bangladesh, collected between 2009 and 2014. Fieldwork in South Sudan was conducted by DM Reeder, ME Vodzak and J Schaer, and bats were sampled during consecutive surveys in Central Equatoria State in 2010 and 2011 and in Central and Western Equatoria States in 2012 and 2013. The Institutional Animal Care and Use Committee of Bucknell University, Pennsylvania, USA and the South Sudanese Ministry for Wildlife Conservation and Tourism approved the fieldwork. In Sierra Leone, bats were sampled in Gola Rainforest National Park by K Matuschewski and N Weber in 2014. Fieldwork was approved by the Forestry Division/Ministry of Agriculture (permit dated March 18, 2014) and the Gola Rainforest Authority. AT Gilbert (National Wildlife Research Center, United States Department of Agriculture, Fort Collins, Colorado, USA) and her team provided a subset of rhinolophid bat samples collected in Kenya in 2009, and KJ Olival provided samples of the bat family Megadermatidae from Bangladesh (sampled in 2011).

2.1.2. Field methods

Ground-height, triple-high and canopy mist-nets, as well as harp traps, were used in combination to assess the local bat species diversity (Kunz and Parsons, 2009). Previous studies have highlighted that bat assemblage compositions differ between ground and canopy level (e.g. Francis, 1994). Several keys were used for bat identifications (Rosevear, 1965; Meester and Setzer, 1971; Koopman, 1975; Csorba et al., 2003; Kingdon et al., 2013) and standard measurements were recorded for every individual, comprising forearm length, body mass and measurements of head and body, tail, ear, hind foot and tibia, to validate species identification. Sex and age class were also recorded (see Supplementary Table S3). Blood samples were taken and preserved as blood dots on filter paper or Whatman® FTA® sample collection cards and thin blood smears were prepared and fixed in 100% methanol. Some bat voucher specimens were collected and deposited in the mammal collection of the National Museum of Natural History in Washington D.C., USA (NMNH) (catalogue numbers are listed in Supplementary Table S3). Individuals of two rhinolophid species from South Sudan could not unequivocally be assigned to a morphospecies based on the standard field measurements as well as measurements of the voucher specimens and skulls. These two species are hereinafter referred to as *Rhinolophus* sp.-1 and *Rhinolophus* sp.-2, respectively.

2.2. Microscopy

Thin blood smears were stained with Romanowsky–Giemsa solution. Light microscopy at a magnification of 1000 X was used to scan all slides for the occurrence of blood parasites. Slides were examined for a minimum of 20 min. Blood smear negative samples were scanned repeatedly to minimise false-negative records. Parasitemia is given as the percentage of parasite-infected erythrocytes in the total number of erythrocytes following the calculation: total number of parasites/product of the mean number of erythrocytes per field * number of counted fields. The mean number of erythrocytes per field was determined by counting one to three fields and the number of parasites was recorded in 20–100 fields and fields with comparable erythrocyte density were chosen. Pictures of infected erythrocytes were processed using Imaging for Windows®. Material from the Garnham collection of the Natural History Museum, London, UK (Supplementary Fig. S1), comprising the two species *N. medusiformis* (Garnham and Heisch, 1953; NHM-registration numbers: 1987:9:1:364-379) and *N. congolensis* (Krampitz and de Faveaux, 1960; Garnham, 1966; NHM-registration numbers: 1987:9:1:380-388), was also examined and photographed with the permission of Dr. Alan Warren. Parasite vouchers have been deposited as follows: slides encoded 'NW' as well as the parasite vouchers from Kenya are accessible through the Museum für Naturkunde – Leibniz Institute for Research on Evolution and Biodiversity (Berlin, Germany; ZMB_EMB_1000-1007); slides encoded 'DMR' from South Sudan are accessioned in the U.S. National Parasite Collection of the National Museum of Natural History (Washington, DC, USA; USNM 1273690-1273701).

2.3. Statistical methods

Statistical significance was assessed using Mann–Whitney test, with a P value of <0.05. All statistical tests were computed with GraphPad Prism 5 (GraphPad Software).

2.4. DNA isolation, PCR amplification and sequencing

DNA was extracted from the dried blood dots and Whatman® FTA® cards (GE Healthcare, USA) using the QIAGEN DNeasy extraction kit (Hilden, Germany). The protocol for animal tissues was performed following the manufacturer's protocol with the minor modification of eluting the samples in 50 μl of AE buffer (Qiagen). PCR was performed using the QIAGEN TopTaq Master Mix with 2–4 μl of genomic DNA as template, and 1 μl of 10 mM of each primer. For molecular characterisation, genes of the three genomes of the parasite were targeted for a robust phylogenetic resolution. The apicoplast caseinolytic protease (*clpc*) and the nuclear adenylosuccinate lyase (*asl*) genes were amplified via nested PCRs using published primers (Martinsen et al., 2008). The nuclear gene elongation factor 2 (*ef2*) was amplified using the primer pair EF2F and EF2R (Schaer et al., 2013). For most samples, the mitochondrial cytochrome *b* (*cytb*) gene was amplified in two parts using the primer pair DW2 and 3932R as well as 3932F and DW4 (Perkins and Austin, 2009), however when both of these primer sets failed for the parasite samples from *Nycteris* hosts, we also attempted different combinations of other established primer sets (e.g. DW1 and DW3 from Creasey et al., 1993; DW6 (AL1413) from Escalante et al., 1998; DW8 from Perkins and Schall, 2002), several newly designed primer sets, as well as whole-mitochondrial genome amplification using the primer sets in Perkins (2008). All primers are listed in Supplementary Tables S4 and S5. All PCR products were sequenced with the amplification primers in both directions using BigDye v3.0 (Applied Biosystems) using the manufacturer's protocol and run on an ABI-373 sequencer.

Parasite sequences included 1140 nucleotides (nt) of the *cytb*, 570 nt of *clpc*, 222 nt of *asl* and 573 nt of the *ef2*-gene (GenBank accession numbers: KP053763, KP053764, KP053765, KP053766, KP053767, KP053768, KP053770, KP053771, KP053772, KP053 773, KP053774, KP053775, KP053776, KP053777, KP053778, KP053779, KP053780, KP053781, KP053782, KP05 3783, KP053 784, KP053785, KP053786, KP053787, KP053788, KP053790, KP 053791, KP053792, KP053793, KP053794, KP053 795, KP053796, KP053797, KP053798, KP053799, KP053800, KP053801, KP05 3802, KP053803, KP053804, KP053805, KP053 806, KP053808, KP053809, KP053810, KP053811 and KP053812; Supplementary Table S6). Although we did obtain sequences for cytochrome oxidase I from the *Nycteris* parasite isolates, for reasons explained in Section 3.3, these were not included in the subsequent molecular phylogenetic analyses.

2.5. Phylogenetic analysis

After Sanger sequencing, primers were trimmed off the sequences before they were assembled and aligned in Geneious 5.4.6 using MUSCLE (Edgar, 2004). The sequence data were combined with the corresponding gene sequences of 42 haemosporidian taxa obtained from GenBank (Supplementary Table S6) comprising the major clades of the Haemosporida. Phylogenetic relationships were evaluated by using maximum-likelihood (ML) and Bayesian inference methods. Data were divided into partitions according to genes and PartitionFinder (Lanfear et al., 2012, 2014) was used to test different partition schemes and DNA substitution models. The best partition schemes and models were used for the phylogenetic analyses. RaxmlGUI (Silvestro and Michalak, 2012) was used for the ML analysis with concatenated alignments and nodal support was evaluated using 100 thorough bootstrap pseudoreplicates (Stamatakis et al., 2008). Bayesian inference was conducted in MrBayes v3.2.0 (Ronquist et al., 2012), with two runs of four chains (three heated, one cold, temperature = 0.20) each for 50 million generations. A GTR + I + Γ type model was used for each independent partition. Reversible rate matrices, partition-specific rate multipliers and stationary state frequencies had a Dirichlet prior. The α and proportion of invariant sites had uniform priors. A prior of all topologies equally likely was used for τ and the prior on branch lengths was set as unconstrained exponential (parameter 10). The first 25% of trees (12,500) were discarded as burn-in.

3. Results

3.1. Identification of Nycteria spp. in candidate bat host genera

We analysed a total of 141 bats from Bangladesh, Kenya, Sierra Leone and South Sudan belonging to six genera, from five chiropteran families: Nycteridae, Rhinolophidae, Hipposideridae, Emballonuridae and Megadermatidae (Table 1). Of these, bat species of only two chiropteran genera, *Rhinolophus* (Rhinolophidae) and *Nycteris* (Nycteridae), were found infected with *Nycteria* parasites, whereas all bat species of the families Hipposideridae (n = 56), Emballonuridae (n = 4) and Megadermatidae (n = 33) were negative for *Nycteria* parasite infections in the study locations at the time of sampling.

In this study, we identified 20 infected bat individuals (Table 1 and Supplementary Table S3). Eight out of 22 (36%) bats of the genus *Nycteris* captured in South Sudan and all four nycterid bats (100%) from Sierra Leone featured infections with *Nycteria* parasites (Fig. 2A). Similarly, four out of 18 (22%) bats of the genus *Rhinolophus* from South Sudan, both (100%) from Sierra Leone, and two out of nine (22%) from Kenya were positive for *Nycteria* parasites (Fig. 2A). All individuals identified as infected with *Nycteria* parasites by microscopy were confirmed by diagnostic PCR and sequence analysis, establishing that there were no false-positive samples. In addition, we tested blood smear-negative samples by PCR for at least two conserved genes for all bats belonging to the Nycteridae, Rhinolophidae and Emballonuridae. In no case did we obtain a molecular signature of potential false-negative *Nycteria* infections. With our PCR-based protocols we detected two samples, which we could not unequivocally assign to single parasite infections (Table 1 and Supplementary Table S3). These potential mixed infections were excluded from the detailed parasitological and molecular analyses.

3.2. Morphology of parasite blood stages and parasitemia

We next quantified the parasitemia of all 18 single infections and included the samples from the Garnham collection (Fig. 2B). As expected, we observed a wide range from patent infections up to gametocytemias of 1.1%. Mean parasitemias were 0.2% (±0.3%) (including the Garnham samples 0.16% (±0.3%)) and 0.01% (±0.01%) (including the Garnham samples 0.04% (±0.03%)) in infected bats of the genera *Nycteris* and *Rhinolophus*, respectively. Differences in parasitemia levels were, however, not significant. Most importantly, gametocyte densities can reach up up to 5×10^6 per ml of blood. The hosts sampled in the late dry season (March–April 2014) in Sierra Leone featured the highest parasitemias (mean 0.5% (±0.48%), whereas the bat hosts, sampled in the wet season in different months between May and October in South Sudan showed mean parasitemias of 0.03% (±0.03%). Our sampling supports the anticipated notion that steady-state gametocytemias vary according to the habitat and season. However, our sample size did not allow for statistical analysis in greater depth and further longitudinal studies are needed.

The morphology of the gametocytes clearly differed between parasites from bats of the genera *Nycteris* and *Rhinolophus* (Fig. 2C). No distinct nuclei were visible in the latter and gametocytes were more diffuse overall. In contrast, gametocytes of parasites of *Nycteris* hosts were well defined and distinct nuclei were visible. The attempt was made to assign the parasites to the described *Nycteria* morphospecies on the basis of the observed morphological characteristics of the blood stages (Fig. 3) and at least four different morphotypes were detected in the *Nycteris* hosts and three in the *Rhinolophus* hosts. These comprise the *Nycteria* parasite species *Nycteria houini, Nycteria eradi, N. congolensis* and *Nycteria gabonensis*. The main diagnostic characters of the investigated *Nycteria* parasite samples are described in Section 3.2.1.

3.2.1. Nycteria parasites isolated from species of the bat host genus Nycteris

The parasite species *N. houini*, isolated from the bat host *Nycteris nana* (DMR 688), featured round and compact gametocytes with circular or oval nuclei that contain a central part with dense homogeneous chromatin (Fig. 3A, B). Up to 17 dark colored pigment granules were distributed irregularly in the cytoplasm. The cytoplasm of female (macro-) gametocytes and male (micro-) gametocytes was granular and deep blue or light blue/gray, respectively.

A distinct characteristic of microgametocytes of *N. eradi*, isolated from the bat host *Nycteris arge* (NW2650, NW2688), was nuclei within a white or pale pink stripe that is free of pigment, which is distributed throughout the cytoplasm and has the appearance of pine needles (Fig. 3C, D). Macrogametocytes are sometimes dotted with small vacuoles containing red spots. The nuclei exhibit a single mass of chromatin with irregular contours. Notably, in some cases the host cell cytoplasm of infected cells stained red, indicating a pH shift in infected erythrocytes.

Table 1
Host genera of *Nycteria* parasites. Listed are all chiropteran genera that have been reported to host *Nycteria* parasites. These genera belong to five families of both suborders of Chiroptera (classification follows Jones and Teeling, 2006). Numbers of all investigated and infected bats of the study are given.

Bat suborder family genus	Yinpterochiroptera			Yangochiroptera	
	Rhinolophidae Rhinolophus	Hipposideridae Hipposideros	Megadermatidae Megaderma	Emballonuridae Taphozous[b]	Nycteridae[a] Nycteris
South Sudan (2010–2013)	4/18	0/18	–	0/4	8/22
Sierra Leone (2014)	2[c]/2	0/38	–	–	4/4
Kenya (2009)	2[c]/9	–	–	–	–
Bangladesh (2011)	–	–	0/33	–	–
Total	8/29	0/56	0/33	0/4	12/26

[a] Nycteridae were classified within the Rhinolophoidea in the past (Simmons, 1998; Simmons and Geisler, 1998).
[b] Two individuals of both *Taphozous* and *Coleura* were investigated.
[c] One sample could not be unequivocally assigned to a single parasite infection, thus the potential mixed infection was excluded from detailed parasitological and molecular analyses.

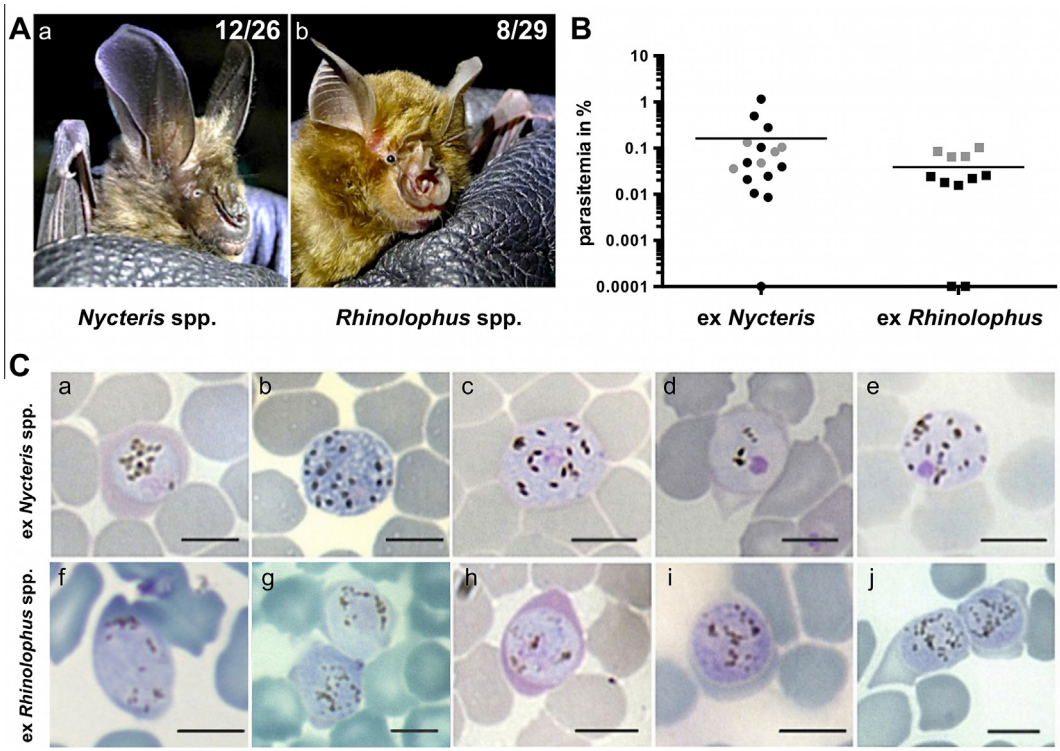

Fig. 2. Parasitological parameters of *Nycteria* parasite infections. (A) Representative hosts of *Nycteria* parasites. Shown are *Nycteris arge* (a) and *Rhinolophus landeri* (b). The prevalence of *Nycteria* parasite infections in nycterid and rhinolophid hosts is indicated in white. (B) Parasitemia as a percentage i.e. the number of gametocyte-infected erythrocytes in total erythrocytes. Samples from this study are highlighted in black, samples from the Garnham collection of the Natural History Museum, London, UK in grey (parasites isolated from *Nycteris capensis* and *Rhinolophus hildebrandtii*). Bar indicates mean parasitemia. Mean parasitemia of parasites from *Nycteris* hosts is 0.16% (±0.29%); from *Rhinolophus* hosts 0.04% (±0.03%) (without Garnham samples: mean parasitemia of parasites from *Nycteris* hosts is 0.20% (±0.35%); from *Rhinolophus* hosts 0.01% (±0.01%)). (C) Representative micrographs (original preparations of the study) showing gametocytes of *Nycteria* parasites from *Nycteris* host species (NW2650 (a, c, d), DMR692 (b), NW2704 (e)) and from *Rhinolophus* host species (967 (f), DMR252 (g, j), NW2664 (ex *Rhinolophus landeri*) (h), DMR257 (i)). "ex" denotes that parasites were isolated from the respective host species or genus. Bars = 5 μm.

The gametocytes in the blood of the host *N. grandis* (NW2704, NW2705; Fig. 3E–H) featured deep blue cytoplasm and irregularly shaped nuclei with single masses of chromatin. In the corresponding microgametocyte the chromatin was denser. Nuclei displayed two alternative patterns; they were either surrounded by pigment or by a white band without pigments. The black pigment that was distributed in the cytoplasm had a granular appearance.

No nuclei were visible and no distinction between macro- and microgametocyte was possible in the parasite that was isolated from *N. thebaica* (DMR 117; Fig. 3I, J), which might be due to weak staining. We note that pigment is often clustered in one area of the periphery of the cytoplasm.

The gametocytes of the parasites of the host species *Nycteris macrotis* (DMR 113, DMR679, DMR692-695; Fig. 3K, L) featured round forms and the cytoplasm had a granular appearance and was blue or light blue colored. In some cases a small compact nucleus was visible. The most characteristic feature in all gametocytes was numerous dark-colored pigment granules that were distributed throughout the cytoplasm, sometimes forming circles or bands and in some cases clustered in the periphery of the

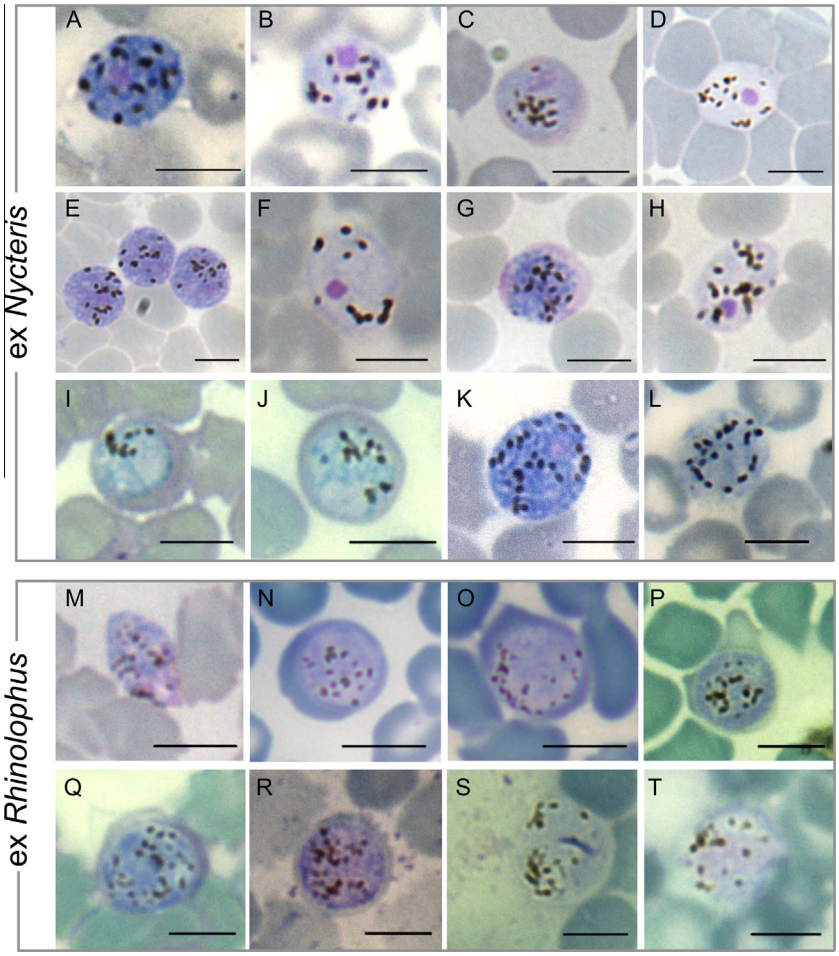

Fig. 3. Representative micrographs of gametocytes of *Nycteria* parasites from *Nycteris* host species and *Rhinolophus* host species. (A, B) macro- and microgametocyte of *Nycteria* cf. *houini* (ex *Nycteris nana,* DMR688), (C, D) macro- and microgametocyte of *Nycteria* cf. *eradi* (ex *Nycteris arge,* NW2688, NW2650), (E, F) macro- and microgametocyte of *Nycteria* sp. (ex *Nycteris grandis,* NW2704), (G, H) macro- and microgametocyte of *Nycteria* sp. (ex *Nycteris grandis,* NW2705), (I, J) gametocytes of *Nycteria* sp. (ex *Nycteris thebaica,* DMR117), (K, L) macro- and microgametocyte of *Nycteria* sp. (ex *Nycteris macrotis,* DMR695, DMR679), (M) microgametocyte of *Nycteria* cf. *congolensis* (ex *Rhinolophus landeri,* NW2668), (N, O) gametocytes of *Nycteria* sp. (ex *Rhinolophus hildebrandtii,* 967), (P, Q) gametocytes of *Nycteria* cf. *gabonensis* (ex *Rhinolophus* sp.-1, DMR252, DMR256), (R) gametocyte of *Nycteria* sp. (ex *Rhinolophus* sp.-1, DMR257), (S, T) gametocytes of *Nycteria* sp. (ex *Rhinolophus* sp.-1, DMR249). "ex" denotes that parasites were isolated from the respective host species or genus. Bars = 5 μm.

cytoplasm. The pigments were round or elongated and relatively large in size.

In conclusion, the parasites from *N. grandis, N. thebaica* and *N. macrotis* could not be unequivocally assigned to any of the known *Nycteria* morphospecies.

3.2.2. *Nycteria parasites isolated from species of the bat host genus Rhinolophus*

The distinct feature of the microgametocytes of *N. congolensis*, isolated from the host species *Rhinolophus landeri* (NW2668; Fig. 3M), was the indistinct and very large nucleus that is well differentiated from the cytoplasm with dark pigment granules. The macrogametocyte featured blue cytoplasm with a smaller nucleus and punctate, large, dark granules that were scattered irregularly throughout the cytoplasm.

The parasite of the host species *R. hildebrandtii* (967; Fig. 3N, O) could not be assigned to a morphospecies. Gametocytes exhibited no apparent nuclear area and, hence, no distinction between micro- and macrogametocyte was possible. Pigment was scattered throughout the light blue cytoplasm.

Several infected erythrocytes in two samples of *Rhinolophus* sp.-1 (DMR252, DMR256; Fig. 2Ce and Fig. 3P, Q) exhibited an irregular polyhedral shape, which is a characteristic feature of the parasite species *N. gabonensis*. The cytoplasm appeared light to dark blue in macrogametocytes and light blue to light pink in microgametocytes, and no distinct nuclear areas were visible. The dark pigment granules in the gametocytes of the parasite that has been found in one *Rhinolophus* sp.-1 (DMR257; Fig. 2Cd and Fig. 3R) show similarities with *N. congolensis*, but the assignment to this parasite species is tentative. Only two gametocytes were found in the infection of the sample DMR 249 (*Rhinolophus* sp.-1) and were therefore insufficient for a morphological characterisation (Fig. 3S, T).

3.3. Molecular phylogeny of *Nycteria* parasites

We attempted to recover sequences for five genes (mitochondrial *cytb* and *cox1*, apicoplast *clpc* and nuclear *ef2* and *asl*) for all *Nycteria* parasites. This was only successful for the parasites that were isolated from *Rhinolophus* hosts. The amplification of *cytb* and *ef2* from parasites that were isolated from *Nycteris* hosts was unsuccessful, even though all established and numerous newly designed primers were tried on the samples as well as the amplification of the whole mitochondrial genome. Sequences for the mitochondrial *cox1* were again easily amplified for parasites of the rhinolophid hosts and were more challenging for the parasites of nycterid hosts. Eventually, we obtained *cox1* sequences for all samples, but sequences from these parasites were evolving at a much faster rate than those from other species and genera in the

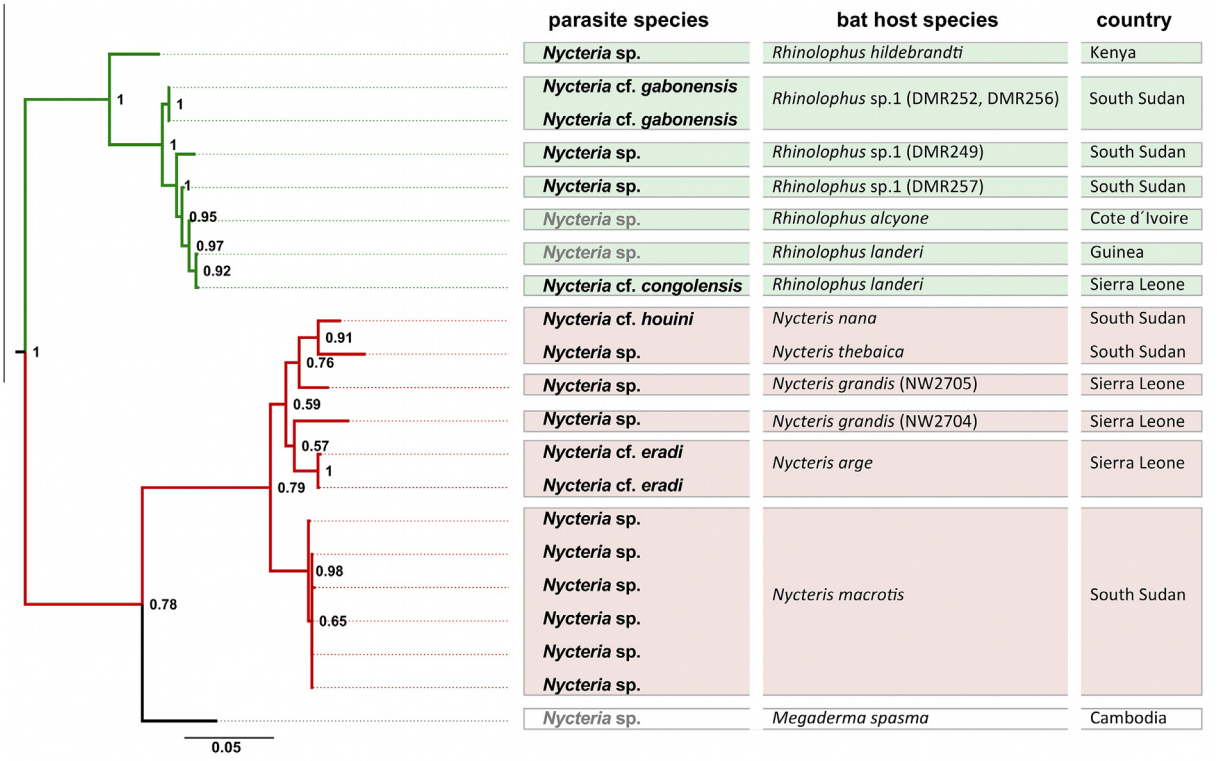

Fig. 4. Two distinct *Nycteria* lineages in rhinolophid and nycterid hosts. Section of the three-genome phylogeny for *Nycteria* parasites recovered from Bayesian analysis. The phylogeny was obtained via analysis of four genes, the nuclear elongation factor 2, nuclear adenylosuccinate lyase, the apicoplast caseinolytic protease and the mitochondrial cytochrome *b*. For each parasite sample, bat host species and sample location are listed and (where possible) the morphologically assigned parasite species. Bayesian posterior probability values are given. Sequences from three previously reported samples, *Nycteria* sp. from *Rhinolophus alcyone, Nycteria* sp. from *Rhinolophus landeri* (Schaer et al., 2013) and *Nycteria* sp. from *Megaderma spasma* (Duval et al., 2007), are indicated in grey. The mitochondrial cytochrome *b* sequence from *Megaderma spasma* was included in the analysis, but nuclear and apicoplast sequences for this *Nycteria* sample were lacking and the apparent proximity to parasites of *Nycteris* hosts can be attributed to the lack of mitochondrial cytochrome *b* from the latter parasite group.

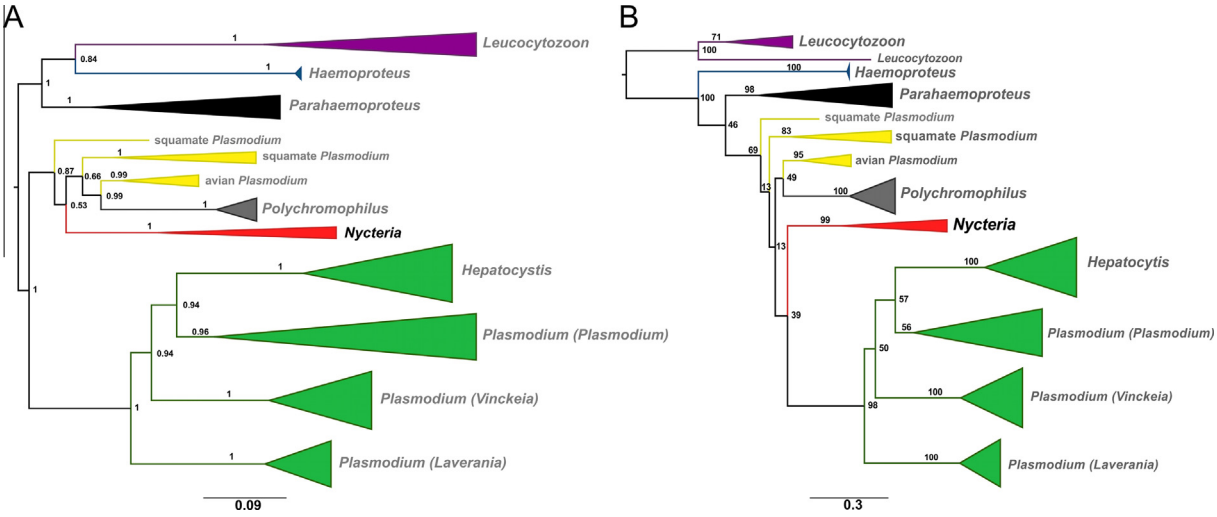

Fig. 5. Molecular phylogeny of *Nycteria* parasites. Three-genome phylogeny for *Nycteria* parasites in the context of the major haemosporidian parasite clades recovered by Bayesian (A) and maximum likelihood analysis (B). Posterior probability values (A) and bootstrap support values (B) are given. The concatenated phylogenies were obtained via analysis of four genes, the nuclear elongation factor 2, nuclear adenylosuccinate lyase, the apicoplast caseinolytic protease and the mitochondrial cytochrome *b*. The main groups are shown as collapsed clades, comprising the mammalian *Plasmodium*/*Hepatocystis* parasite clades, the chiropteran parasite genus *Polychromophilus*, the avian/squamate parasite genera and the genus *Nycteria*. (A) Placement of *Nycteria* parasites as sister to a clade that contains the lizard and bird *Plasmodium* spp. as well as *Polychromophilus* parasites with modest support (87% posterior probability) obtained via relaxed molecular clock Bayesian methods and no predefined root. (B) Maximum likelihood analysis and rooting of the best tree with species of *Leucocytozoon* places the *Nycteria* parasite clade as sister to the mammalian *Plasmodium* spp., but with very weak support (39%).

family (Supplementary Fig. S2). The branch leading to the *Nycteria* parasite sequences that were isolated from *Nycteris* bats was 65% longer than any other branch in the tree. For this reason, all *cox1* sequences were excluded from the final phylogenetic analyses.

Both phylogenetic analyses clearly grouped *Nycteria* parasites into a well-supported (99% bootstrap and 100% posterior probability) monophyletic clade. Within this clade, however, the *Nycteria* parasites obtained from *Nycteris* and *Rhinolophus* bats separated into two distinct, well-supported clades (Fig. 4), corresponding to the two genera of hosts. Also, our analysis did not reveal any geographic relationships despite the large distance between the western and eastern African sampling sites and sympatry of infected *Nycteris* and *Rhinolophus* hosts in both locations. The *cytb* sequence from the parasite isolated from *M. spasma* has been included in the analyses, but cannot be unequivocally assigned to either parasite group (Fig. 4). We note that the conflicting apparent proximity to parasites of *Rhinolophus* hosts in the phylogenetic tree that has been recovered from the ML analysis and to parasites of *Nycteris* hosts in the Bayesian analyses can be attributed to the lack of *cytb* from the latter parasite group.

The placement of *Nycteria* parasites among the major haemosporidian groups differed between analyses (Fig. 5). Bayesian analyses grouped both *Nycteria* spp. and *Polychromophilus* spp. within the clade of saurian and avian *Plasmodium* spp., with modest support (Fig. 5A, 87% posterior probability), whereas ML analysis of the dataset and rooting of the best tree with parasites of the genus *Leucocytozoon* as suggested by other phylogenetic studies (Perkins and Schall, 2002; Martinsen et al., 2008), placed the *Nycteria* parasite clade as sister to the mammalian *Plasmodium* spp., albeit with very weak support (39%) (Fig. 5B).

4. Discussion

Following screening of individuals from the candidate bat families Megadermatidae, Emballonuridae, Hipposideridae, Rhinolophidae and Nycteridae, infections by *Nycteria* parasites were only found in the latter two families. Six out of the seven sampled *Nycteris* spp. harboured parasites, as did three out of five *Rhinolophus* spp.. Within the Megadermatidae the genus *Megaderma* comprises only two species, both with distributions across southeastern and southern Asia. *Nycteria* parasites have been reported from *M. spasma* in Cambodia (Duval et al., 2007; Schaer et al., 2013). We sampled individuals of the species *Megaderma lyra* but did not observe any parasites in these congeneric Asian bats. Records of *Nycteria* parasites from hipposiderid hosts are limited to the Asian bat species *H. larvatus* and the African bat species *H. ruber,* whereas the hipposiderid samples of this study belonged to African species only. Within the Emballonuridae, only the species *T. perforatus* was reported to host *Nycteria* parasites; the current study investigated just two samples from *Taphozous mauritianus* and *Coleura afra,* in which no parasites were detected. We therefore consider it likely that future systematic cross-sectional studies, as described herein, will reveal additional *Nycteria* bat hosts, which are not represented in the present study. Nevertheless, *Nycteria* parasite infections were readily retrieved at three distant locations by capturing rhinolophid and nycterid bats. Together with the observed high prevalences in these bat hosts we speculate that infections with species of *Nycteria* parasites are likely most abundant in species of these two bat families.

Our morphological analysis permitted placement of the infections into the two proposed *Nycteria* parasite groups (Rosin et al., 1978), i.e. *Nycteria* parasites of *Rhinolophus* hosts as the "diffuse type" (round gametocytes with indistinct nuclei) and parasites of *Nycteris* hosts as "insignia" group (round gametocytes with distinct nuclei). This allowed us to assign molecular data to the described gametocyte morphospecies as well as host species. Beyond this positioning, *Nycteria* morphospecies of the *Rhinolophus* hosts are almost indistinguishable in the blood, since they differ more in their exoerythrocytic schizonts (Rosin et al., 1978). We note that we cannot corroborate previous reports on parasites of the species *N. medusiformis*, which were described to induce characteristic filaments that protrude from the surface of the infected erythrocyte and reach considerable lengths of up to 4 μm. It was already noted that these filaments are likely degenerative formations of the erythrocyte and not cellular structures of the parasite itself (Garnham and Heisch, 1953; Krampitz and de Faveaux, 1960), and future studies are warranted to explore whether gametocytes of *Nycteria* parasites remodel host erythrocytes in a distinct manner.

One limitation of the present capture-release study is exclusive sampling of parasite blood stages and the obvious lack of the important tissue stages for species identification. This is largely overcome by our phylogenetic analysis that strongly supports an assignment of *Nycteria* lineages on the basis of host genera and, in many cases, even at species level, since most parasites from the same host species were identical or grouped closely together. Five lineages clearly grouped separately to the assigned species in our phylogenetic analysis (Fig. 4). In support of these molecular signatures, the gametocytes display distinct morphologies that do not match the known descriptions. Assigning these *Nycteria* parasites, which were isolated from the bat species *N. macrotis*, *Nycteris grandis*, *N. thebaica*, *R. hildebrandti* and *Rhinolophus* sp.-1 to novel species is, however, premature. Clearly, further detailed morphological information from other life cycle stages and additional molecular data are needed. Yet, our analysis provides compelling evidence that the *Nycteria* spp. complex might comprise more species than described in the literature.

Our data show that the average parasitemia of *Nycteria* parasite infections reaches ~0.2%, indicating that robust gametocytemia can frequently be achieved in *Nycteria* parasite infections. *Plasmodium*-like liver stages, which likely produce only several thousand merozoites, combined with the absence of erythrocytic parasite propagation, suggest that transmission is probably continuous. One plausible explanation is frequent sporozoite inoculations by regular exposure to *Nycteria*-infectious vectors. Although less likely, gametocytes of *Nycteria* parasites might persist for extended periods in the peripheral blood. Together with our observation of low to very high prevalences, ranging from 22% to 100% of all captured host species, we propose that *Nycteria* parasite transmission is very efficient in the absence of parasite population expansion inside erythrocytes, indicative of a successful alternative strategy employed by *Nycteria* parasites. This differs fundamentally from *Plasmodium* parasites that undergo extended erythrocytic merogony resulting in clinical malarial episodes.

Our inability to amplify either cytochrome *b* or whole mitochondrial products from *Nycteria* parasites from the *Nycteris* spp. hosts, combined with the observed differences in rate of evolution of the *cox1* gene in these parasites, is puzzling and raises the possibility that these *Nycteria* spp. may have either lost their mitochondrial genome or that it has become even more reduced than that of other haemosporidian parasites. While nuclear mitochondrial DNA (numts) are very rare in *Plasmodium* spp. (Richly and Leister, 2004), it is possible that the *cox1* amplicons were derived from those copies. There is precedence for such an observation from avian haemosporidian parasites (Zehtindjiev et al., 2012). In their study, the authors used established primers and protocols as well as newly designed primers to amplify the mitochondrial cytochrome *b* gene of *Plasmodium polymorphum* in a sample that was co-infected with a light infection of *Plasmodium relictum.* They easily detected the latter parasite species, however the PCR-based assays failed to amplify *P. polymorphum*. The authors list co-infections and primer issues as possible explanations for the lack of the gene amplification, but also speculate about the possibility of the loss of the *cytb* gene in *P.*

polymorphum. The apparent failure to amplify *cytb* sequence information in some haemosporidian lineages warrants further explorations of their mitochondrial genomes and morphology.

An intriguing question is whether the origin of *Nycteria* parasites can be explained by host switch or if the positioning of the parasite genus in the haemosporidian parasite phylogeny rather reflects an ancient lineage that might have given rise to the mammalian *Plasmodium/Hepatocystis* parasite clade. Our earlier data (Schaer et al., 2013) supported the latter hypothesis. However, the recent study revealed an alternative scenario, where *Nycteria* parasites share a closer relationship with the genus *Polychromophilus* and parasites of birds and squamates. Clearly, the current dataset lacks power to effectively resolve the basal relationships between genera in the family and additional nuclear markers will perhaps clarify the recent findings.

Apart from the phylogenetic placement of the genus *Nycteria* among all major haemosporidian clades, the separation of *Nycteria* parasites according to host bat genus was verified and confirms the finding of Rosin et al. (1978), which was based on morphological data. Strikingly, no patterns of geographic origin were apparent, strongly suggesting phylogenetically ancient parasite/host congruence. Furthermore, most parasites grouped by host species, indicating a high specificity, i.e. closely related host species harbor similar parasites.

Bats (Chiroptera) are currently classified in the two suborders Yangochiroptera and Yinpterochiroptera that comprise nineteen extant families (Jones and Teeling, 2006). Interestingly, the chiropteran *Plasmodium* and *Hepatocystis* parasite species are apparently restricted to two host families of Yinpterochiroptera, whereas *Polychromophilus* parasites are adapted to two bat families of Yangochiroptera. In marked contrast, the haemosporidian genus *Nycteria* is present in five different bat families, from both chiropteran suborders (Table 1). The molecular analysis in this study highlights the robust grouping of *Nycteria* parasites from rhinolophid (Yinpterochiroptera) and nycterid hosts (Yangochiroptera) in one distinct clade. However within this clade, parasites group according to host genus. Host switches into distantly related bat families/genera might have been followed by speciation/diversification. Further studies of parasites of emballonurid and megadermatid hosts are expected to add critical information to the speciation and origin of *Nycteria* parasites.

The bat families Rhinolophidae and Nycteridae that were identified to host *Nycteria* parasites in the present study are monotypic families with a wide variety of taxa (Simmons, 2005). Until recently, both bat families have been placed in the superfamily Rhinolophoidea together with Megadermatidae and Hippo sideridae based on morphological data (Simmons, 1998; Simmons and Geisler, 1998). However, molecular studies (e.g. Teeling et al., 2005) suggested that the chiropteran family Nycteridae belongs to the suborder Yangochiroptera. This raises important questions of the commonality of distantly related bat hosts and the adaptations of *Nycteria* parasites. With the notable exception of the bat species *N. grandis,* which occasionally preys on small vertebrates, the other species in both families are mainly insectivorous. They forage by using echolocation and the day roosts of the African species range from caves and cave-like structures to hollow trees and logs. Overall, these bats are less arboreal than the bat hosts of *Hepatocystis* parasites and mostly use understory habitats below the canopy level. Most species are solitary or live in small groups, but some, e.g. *R. landeri, R. hildebrandti* and *N. thebaica*, also form colonies of several hundred individuals. Interestingly, it was noted early on that sometimes several insectivorous bat species (including *Nycteria* hosts) roost together in caves (Garnham and Heisch, 1953). African Rhinolophidae and Nycteridae inhabit a broad variety of habitats, however many species at least partly occur in the forest zone and it is plausible that habitat preference is an important signature to restrict candidate vectors that transmit *Nycteria* parasites.

The identity of the vector is an important missing piece in the life cycle of *Nycteria* parasites. It was suggested that the vector is an arthropod other than a mosquito (Garnham and Heisch, 1953). This is verified for parasite species of the haemosporidian genus *Polychromophilus*, which are vectored by nycteribiid flies, obligate ectoparasites of bats (Corradetti, 1936; Mer and Goldblum, 1947). Ectoparasites that have been reported from a variety of African *Nycteris* and *Rhinolophus* spp. include bat flies (Diptera: Nycteribiidae and Streblidae), bed bugs (Hemiptera: Polyctenidae and Cimicidae), fleas (Siphonaptera: Pulicidae and Ischnopsyllidae) and ticks/mites (Arachnida: Acarina). Previous attempts to identify the vector of *Nycteria* parasites were unsuccessful (e.g. Rodhain, 1926; McGhee, 1949; Garnham, 1951). Most likely the vectors of *Nycteria* parasites belong to the family Diptera, as all investigated species of haemosporidian parasites are transmitted exclusively by blood-sucking dipteran insects. Culicidae, Ceratopogonidae and other biting dipterans or temporary ectoparasites are all potential vector candidates.

In conclusion, our present findings highlight that studies on neglected haemosporidian parasites might offer previously unrecognised insights into evolution of human pathogens and into diverse parasite strategies to achieve stable transmission to host populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2015.01.008.

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