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Authors

Karadjian, Gregory
Hassanin, Alexandre
Saintpierre, Benjamin
et al.

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Highly rearranged mitochondrial genome in *Nycteria* parasites (Haemosporidia) from bats

Gregory Karadjian^{a,1,2}, Alexandre Hassanin^{b,1}, Benjamin Saintpierre^c, Guy-Crispin Gembu Tungaluna^d, Frederic Arie^e, Francisco J. Ayala^{f,3}, Irene Landau^a, and Linda Duval^{a,3}

^aUnité Molécules de Communication et Adaptation des Microorganismes (UMR 7245), Sorbonne Universités, Muséum National d'Histoire Naturelle, CNRS, CP52, 75005 Paris, France; ^bInstitut de Systématique, Evolution, Biodiversité (UMR 7205), Sorbonne Universités, Muséum National d'Histoire Naturelle, CNRS, Université Pierre et Marie Curie, CP51, 75005 Paris, France; ^cUnité de Génétique et Génomique des Insectes Vecteurs (CNRS URA3012), Département de Parasites et Insectes Vecteurs, Institut Pasteur, 75015 Paris, France; ^dFaculté des Sciences, Université de Kisangani, BP 2012 Kisangani, Democratic Republic of Congo; ^eLaboratoire de Biologie Cellulaire Comparative des Apicomplexes, Faculté de Médecine, Université Paris Descartes, Inserm U1016, CNRS UMR 8104, Cochin Institute, 75014 Paris, France; and ^fDepartment of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697

Contributed by Francisco J. Ayala, July 6, 2016 (sent for review March 18, 2016; reviewed by Sargis Aghayan and Georges Snounou)

Haemosporidia parasites have mostly and abundantly been described using mitochondrial genes, and in particular cytochrome *b* (*cytb*). Failure to amplify the mitochondrial *cytb* gene of *Nycteria* parasites isolated from Nycteridae bats has been recently reported. Bats are hosts to a diverse and profuse array of Haemosporidia parasites that remain largely unstudied. There is a need to obtain more molecular data from chiropteran parasites. Such data would help to better understand the evolutionary history of Haemosporidia, which notably include the *Plasmodium* parasites, malaria's agents. We use next-generation sequencing to obtain the complete mitochondrial genome of *Nycteria* parasites from African *Nycteris grandis* (Nycteridae) and *Rhinolophus alcyone* (Rhinolophidae) and Asian *Megaderma spasma* (Megadermatidae). We report four complete mitochondrial genomes, including two rearranged mitochondrial genomes within Haemosporidia. Our results open outlooks into potentially undiscovered Haemosporidian diversity.

cytochrome *b* | evolution | phylogeny | *Nycteris*

Haemosporidia blood parasites (phylum Apicomplexa) are vector-borne protozoan parasites that infect multiple vertebrate hosts such as squamates, chelonians, birds, and mammals, including humans, in which five species of the genus *Plasmodium* are known to cause malaria (1, 2). The diversity of Haemosporidia has been mostly investigated using mitochondrial genes. In particular, the mitochondrial (mt) cytochrome *b* (*cytb*) gene is one of the most widely used genetic markers to characterize the diversity of Haemosporidia parasites, and has given rise to many phylogenetic reconstructions to document the evolutionary history of the Haemosporidia (3–5). All Haemosporidia mt genomes previously sequenced share the same structure, characterized by a tandemly repeated linear element of ~6 kb containing the three protein-coding genes *cytb*, cytochrome *c* oxidase subunit 1 (*cox1*), and cytochrome *c* oxidase subunit 3 (*cox3*) and two highly fragmented (small and large subunits) ribosomal RNA (rRNA) genes (6). In the *Plasmodium* species, copy numbers of the mt genome are dozens to hundreds of times more numerous than the nuclear genomes (7, 8). Consequently, mt genes are generally easily amplified using traditional PCR techniques.

Nevertheless, several cases of unsuccessful PCR amplification of mt DNA markers have been recently reported, in particular for *Nycteria* and *Plasmodium* parasites infecting, respectively, Nycteridae bats and Alaudidae birds (9, 10). Three alternative hypotheses have been proposed to explain these negative results: (i) loss of the mitochondrial genome during the evolution of these parasites; (ii) transfer of some mitochondrial genes into the nuclear genome; and (iii) technical problems due to the use of inefficient or misdesigned primer sets.

Bats are hosts to a large, diverse, and specific array of Haemosporidia parasites (11). However, this parasite diversity remains largely unstudied at the molecular and genomic levels,

and this lack of knowledge limits the understanding of the evolutionary history of Haemosporidia, in particular their basal diversification.

Nycteria parasites have been primarily described, based on traditional taxonomy, in African insectivorous bats of two families, Nycteridae and Rhinolophidae (12). The type species of the genus *Nycteria* is *Nycteria medusifformis*, first described from Kenya by Garnham and Heisch in 1953 (13) from the Egyptian slit-faced bat (*Nycteris thebaica*). Later, this species, *N. medusifformis*, was reported in other species of *Nycteris*, including the large slit-faced bat, *Nycteris grandis* (14). A total of six species have so far been described in the genus *Nycteria* (14).

To contribute to the identification and characterization of *Nycteria* parasites in bats and to ascertain the reasons for the failure of PCR amplification in previous studies, we have used deep next-generation sequencing (NGS) to assemble the complete mitochondrial genome of four different *Nycteria* species: *N. medusifformis* and *Nycteria* sp. (mixed infection) from *N. grandis* (Nycteridae); *Nycteria gabonensis* from *Rhinolophus alcyone* (Rhinolophidae); and a species of *Nycteria*, herein described and named *Nycteria heischi* n. sp., from *Megaderma spasma*

Significance

Understanding the evolutionary history of Haemosporidian parasites would help to understand human malaria evolution. Nevertheless, Haemosporidia parasite diversity in bats remains largely unstudied. In addition, some cases of unsuccessful PCR amplification of cytochrome *b*, the most widely used molecular marker to characterize Haemosporidia parasites, have recently been reported for *Nycteria* parasites infecting Nycteridae bats. Here we used next-generation sequencing to characterize mitochondrial genomes of parasites from the *Nycteria* genus. Our results have revealed an unsuspected mitochondrial genome rearrangement within Haemosporidia.

Author contributions: I.L. and L.D. designed research; G.K., A.H., G.-C.G.T., I.L., and L.D. performed research; A.H., G.-C.G.T., and L.D. collected blood from bats; G.K., A.H., B.S., F.A., F.J.A., I.L., and L.D. analyzed data; and G.K., A.H., B.S., F.A., F.J.A., I.L., and L.D. wrote the paper.

Reviewers: S.A., Yerevan State University; and G.S., UPMC Sorbonne Universités.

The authors declare no conflict of interest.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession nos. [KX090645](https://doi.org/10.1093/oxfordjournals.oxford.a0173113), [KX090646](https://doi.org/10.1093/oxfordjournals.oxford.a0173113), [KX090647](https://doi.org/10.1093/oxfordjournals.oxford.a0173113), and [KX090648](https://doi.org/10.1093/oxfordjournals.oxford.a0173113)).

¹G.K. and A.H. contributed equally to this work.

²Present address: Unité Biologie Moléculaire et Immunologie Parasitaire (UMR 956), Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail–École Nationale Vétérinaire d'Alfort–Institut National de la Recherche Agronomique, 94701 Maisons-Alfort Cedex, France.

³To whom correspondence may be addressed. Email: fjyala@uci.edu or lduval@mnhn.fr.

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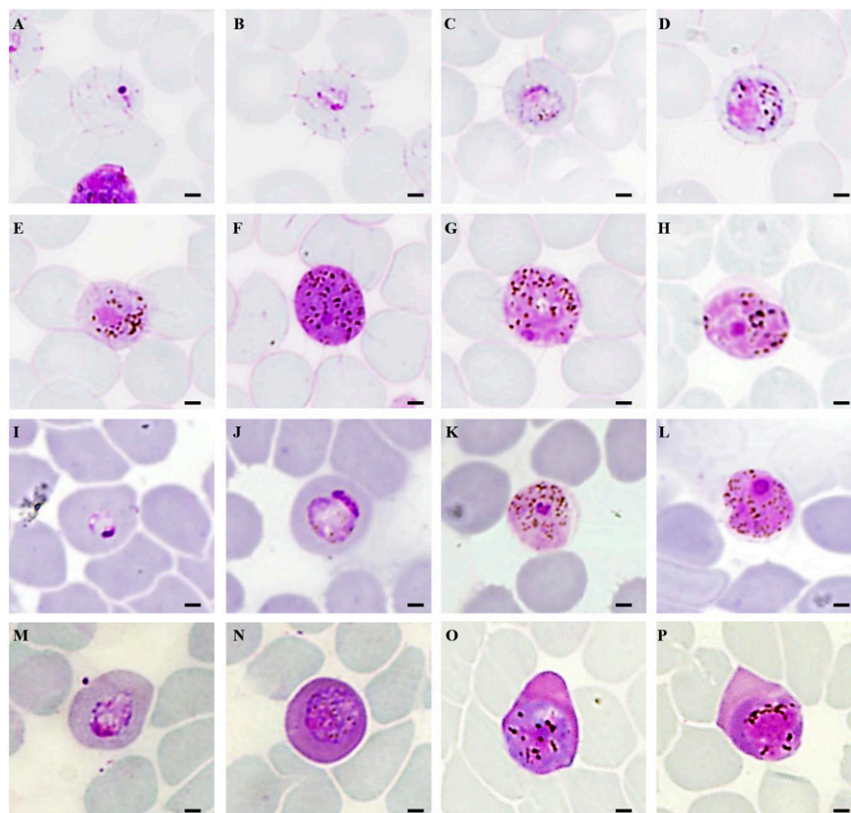


Fig. 1. Microphotographs of gametocytes of *Nycteria* parasites from *N. grandis* (A–H), *M. spasma* (I–L), and *R. alcyone* (M–P). (A and B) Very young gametocytes. (C–E) Immature gametocytes. (F and G) Macro- and microgametocytes. (H) Old gametocyte of *N. medusifformis* from *N. grandis* (273VI). (I and J) Immature gametocytes. (K and L) Macro- and microgametocytes of *N. heischi* n. sp. from *M. spasma* (C289). (M and N) Immature gametocytes. (O and P) Macro- and microgametocytes of *N. gabonensis* from *R. alcyone* (289VI). (Scale bars, 1.5 μm .)

(Megadermatidae). Our results have revealed an unsuspected mitochondrial genome rearrangement within the Haemosporidia.

Results

Three out of 13 *N. grandis* and four out of four *R. alcyone* from a total of 195 bats (six families) were found to be infected with *Nycteria* parasites in the Democratic Republic of Congo. Four out of five *M. spasma* from a total of 90 bats (six families) were found to be infected with *Nycteria* parasites in Cambodia.

All of the three *N. grandis* were coinfecting with *N. medusifformis* (13) and an unidentified *Nycteria* sp. The four *R. alcyone* were infected with *N. gabonensis* (14), *N. medusifformis* and *N. gabonensis* were identified according to their morphological traits, from *N. grandis* and *R. alcyone*, respectively (Fig. 1). Thin blood smears of *N. medusifformis* from *N. grandis* [Museum National d'Histoire Naturelle (MNHN) registration nos. 178PBP, 225PB, and 226PB] and *N. gabonensis* from *Rhinolophus sylvestris* (MNHN registration nos. 155GG, 376GG, and 377GG), collected respectively in 1977 and 1976–1977 in Gabon, from the Landau collection stored at the Museum National d'Histoire Naturelle, were included in the study for taxonomic examinations. The *Nycteria* parasites from *M. spasma* were characterized and described morphologically and named *N. heischi* (MNHN registration no. 206YZ; hapantotype collection no. PXX99).

The morphological characteristics of *N. medusifformis* gametocytes were consistent with the original description (12, 13). Mature gametocytes completely filled the red blood cells (RBCs), which were slightly enlarged (8 μm instead of 6 μm for uninfected RBCs; $n = 10$). In most stages of gametocytes, filaments could be observed at the surface of infected erythrocytes. These filaments became fewer and shorter as the gametocytes matured, and sometimes disappeared at the later stages. Pigment grains were fine and

irregularly scattered throughout the cytoplasm. Macrogametocytes were highly chromophilic and appeared dark blue-purple with small and condensed nuclei. Microgametocytes had a pale pink color and the nuclei displayed a small central condensation of chromatin reminiscent of a “cocarde” (rosette) (Fig. 1 A–H). The *Nycteria* sp. morphological type, observed in *N. grandis* in a mixed infection, was similar to *N. medusifformis*. Nevertheless, it did not present the highly characteristic filaments observed in *N. medusifformis*. This morphotype differed mostly in size, in the nucleus of the microgametocytes, and in pigment features. However, the number of gametocytes of this *Nycteria* sp. was insufficient for a full description. *N. medusifformis* was highly predominant, whereas *Nycteria* sp. was less frequent.

With NGS sequencing, we obtained two complete mitochondrial genomes of *Nycteria* from *N. grandis* (273VI), using iSeGWalker. No other *Haemosporidia* mt genomes were detected in this bat sample by NGS sequencing. Both mt genomes were 6,091-bp-long, with a total of 113,661 reads and a mean coverage of 1,006. These two *Nycteria* parasites share the same genome organization, and their nucleotide sequences differ in only 83 out of the 6,091 positions (about 1.4%). Read coverages of 80% and 20% were obtained for the two *Nycteria* mt genomes. Based on the morphological observation frequencies of both *Nycteria* morphotypes, we propose assigning the predominant mt genome to *N. medusifformis* and the less frequent to *Nycteria* sp.

Both *N. medusifformis* and *Nycteria* sp. mt genomes contained the three protein-coding genes *cytb*, *cox1*, and *cox3* as well as the two highly fragmented rRNA genes (Fig. 2B). They displayed a highly rearranged mt genome organization compared with other *Nycteria* parasites and all other Haemosporidia mt genomes sequenced until now. These rearrangements concerned gene order,

transcriptional direction (gene inversion at *cox3*), as well as the pattern of high fragmentations of the large- and small-subunit rRNA genes (Fig. 2B). However, the rRNA sequences were well-conserved between the *Nycteria* parasites from *N. grandis* and all other known Haemosporidia mt genome parasites. The three protein-coding genes, *cox1*, *cytb*, and *cox3*, were divergent from those of *N. gabonensis* and *N. heischii* and from all other Haemosporidia parasites.

N. medusifformis mt genome reconstruction was successfully validated by the amplification of a 1,439-bp mt fragment using classical PCR tools with specifically designed primers followed by Sanger sequencing. However, we did not succeed in amplifying and sequencing the mt fragment of *Nycteria* sp. using classical PCR and Sanger sequencing. This classical method favored the amplification and/or sequencing of only the *N. medusifformis* parasite in the case of the *N. grandis* mixed infection (15).

Only one species of *Nycteria* was observed in thin blood smears from *R. alcyone*. Morphological characteristics were consistent with the original description of *N. gabonensis* gametocytes (14) (Fig. 1 M–P). Briefly, the erythrocyte was deformed and enlarged (mean measurements of 9 μm ; $n = 10$). It displayed a more or less dark pink color. Mature gametocytes (mean measurements of 7.65 μm ; $n = 10$) were roundish and did not fill the erythrocyte. Microgametocytes had a large well-limited nucleus with a diffuse chromatin and dark pigments, which were mostly seen in the periphery of the nucleus. The macrogametocyte nuclei were smaller and irregular in shape. Only one mitochondrial genome of 5,999 bp was reconstructed from the *R. alcyone* (289VI) sample, with a total of 16,361 reads and a mean coverage of 272. This mt genome could confidently be assigned to *N. gabonensis*.

***N. heischii* from *M. spasma*.** The shape and staining affinities of the *M. spasma* RBCs were not modified by the presence of the *N. heischii* gametocytes (Fig. 1 I–L). Young gametocytes were ovals and occupied about a quarter of the RBCs. The nucleus was made of two or three chromatin grains of variable size, often located between a bluish relatively large vacuole and a smaller white one. A few very fine grains of pigment could be observed in the cytoplasm. As gametocytes matured, they became rounded. The cytoplasm was light blue or grayish and the nucleus was granular and peripheral, either half-moon-shaped in younger stages or larger in

older stages. Mature gametocytes of both sexes were pink, of a light color in macrogametocytes and much darker in microgametocytes. They filled the RBCs almost completely, which were slightly enlarged (mean measurements of about 7 μm ; $n = 10$). There was no “accessory chromatin dot.” The nucleus of the microgametocytes was arranged as a rosette (*en cocarde*) comprising a pink more or less homogeneous area devoid of pigment and a central condensed granular zone. The pigment (about 30 grains) was fine, of variable shape and size, and dispersed in the cytoplasm. The nucleus of the macrogametocytes was smaller, dense, and granular. The cytoplasm was slightly foamy and the pigment was coarser and more abundant than in the microgametocytes.

N. heischii was morphologically related to the group of African *Nycteria* from *Nycteria* bats with the nucleus of the gametocytes *en cocarde*: *N. medusifformis* (13), *Nycteria houini*, and *Nycteria erardi* (14). The schizogonic stages of *N. heischii* are unknown. Gametocytes differ morphologically from (i) *N. medusifformis* by the absence of filaments on the erythrocyte; (ii) *N. houini* by their effect on RBCs, which stain a dark-pink color in the latter (i.e., *N. houini*) but are colorless when seen in *N. heischii*, and by the “rice grains” aspect of the pigment, scarcer and irregularly disposed in *N. houini* and abundant, dispersed, and in small grains in *N. heischii*; and (iii) *N. erardi* by the “pine needle” pigment, scarcer, irregularly disposed, and abundant, yet dispersed and in small grains in *N. heischii*.

Taxonomic Summary.

Taxon: *N. heischii* n. sp. (Karadjian, Landau, and Duval, 2016).

Type host: *M. spasma* (Linnaeus, 1758).

Type locality: Mondolkiri, Cambodia.

Etymology: Named in honor of R. B. Heisch, codiscoverer with P. C. C. Garnham of *N. medusifformis*, the type species of the *Nycteria* genus.

Hapantotype: One blood film deposited at the collection of the Museum National d’Histoire Naturelle de Paris (collection no. PXX99).

The complete mitochondrial genome of 5,989 bp from *N. heischii* (C289) was reconstructed with a total of 64,660 reads and a mean coverage of 734. Both mt genomes of *N. gabonensis* and *N. heischii*, obtained respectively from *R. alcyone* (289VI) and *M. spasma*

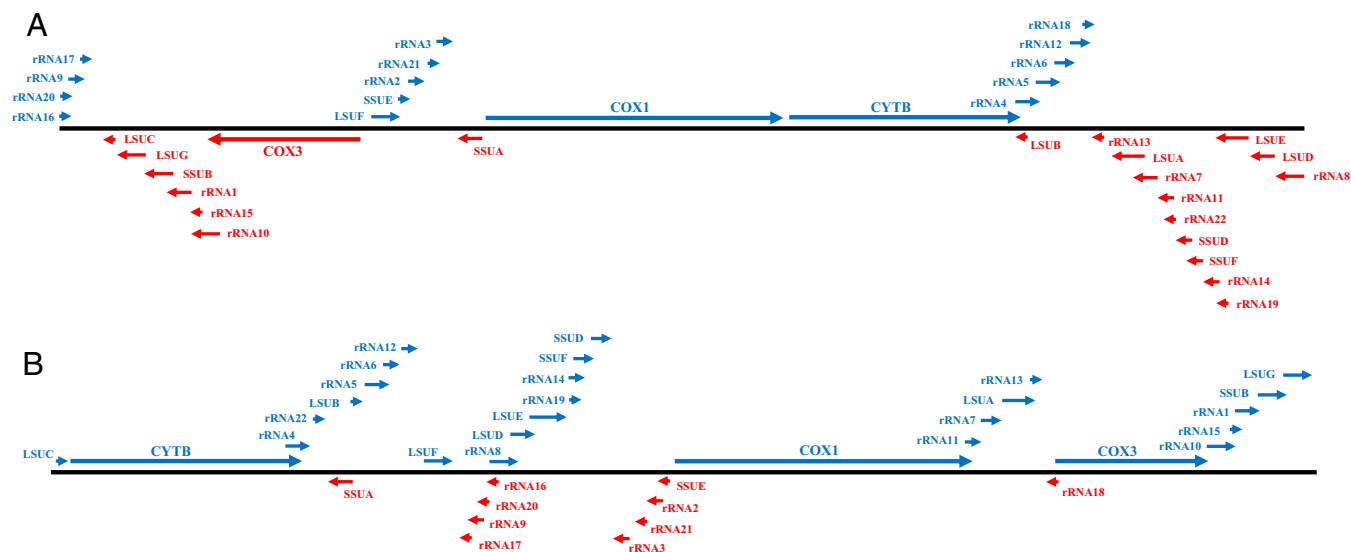


Fig. 2. Two schematic types of mitochondrial genome organization. (A) Typical Haemosporidia mt genome organization, as in *N. gabonensis* and *N. heischii*. (B) *N. medusifformis* and *Nycteria* sp. mt genome organization. LSU, large subunit; SSU, small subunit.

(C289), show the same genome organization as that of all other species of Haemosporidia parasites currently known (Fig. 2A).

GenBank accession numbers of the four *Nycteria* mt genomes are *N. medusififormis* (273VI), KX090645; *Nycteria* sp. (273VI), KX090646; *N. gabonensis* (289VI), KX090647; and *N. heischii* (C289), KX090648.

Phylogenetic analyses based on the datasets (3PCG and *cytb*) supported the monophyly of the genus *Nycteria* [posterior probabilities (PP) > 0.90] as well as a sister-group relationship between *N. medusififormis* and *Nycteria* sp. from *N. grandis* (PP = 1) (Fig. 3 and Fig. S1). The branch leading to the two latter species is much longer than other branches of the tree.

The placement of the genus *Nycteria* in deep-level relationships within the Haemosporidia could not be confidently determined.

Discussion

The use of the deep NGS approach has allowed us to discover an unsuspected and highly rearranged mitochondrial genome in *Nycteria* parasites infecting African bats of the family Nycteridae,

which had not been successfully amplified and sequenced using classical PCR and Sanger sequencing approaches. Our results reveal that neither the order nor the transcriptional direction of the three protein-coding mitochondrial genes (*cytb*, *cox1*, and *cox3*) is conserved within the *Nycteria* genus and Haemosporidia parasites. The large and small subunits of the rRNA gene sequences are well-conserved, but their highly fragmented patterns in *N. medusififormis* and *Nycteria* sp. are remarkably different from all other Haemosporidia mt genomes sequenced until now (8). This rearrangement of the mt genomes of *N. medusififormis* and *Nycteria* sp. was discovered by means of deep NGS sequencing and primer sets. In other Apicomplexan parasites outside the order Haemosporidia, such as the order *Piroplasma* (genera *Babesia* and genera *Theileria*), the mt genomes exhibit remarkably diverse structures (6). However, the biological and evolutionary significance of this mt genome diversity remains to be explored (6).

The use of deep NGS sequencing associated with morphological descriptions might also be an alternative and reliable

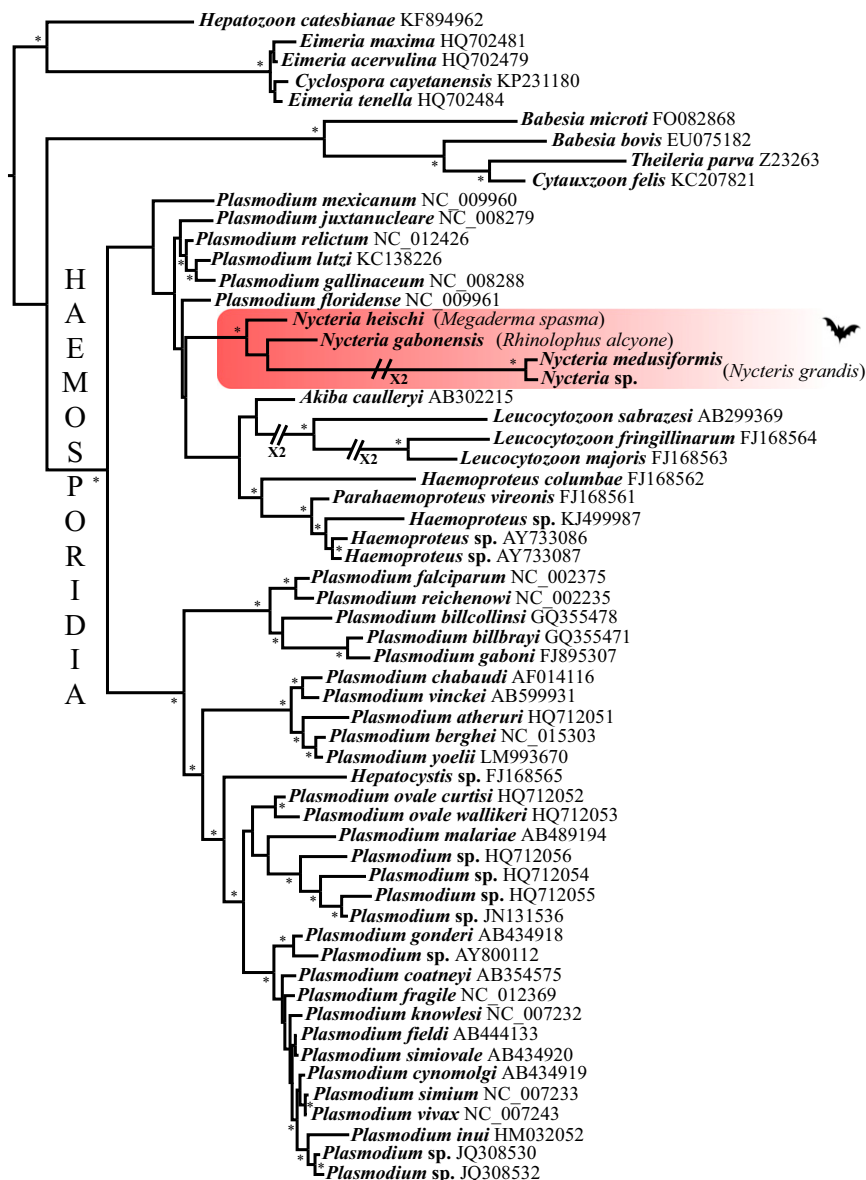


Fig. 3. Mitochondrial genome phylogeny (3PCG) of Haemosporidia parasites including the four *Nycteria* species obtained by MrBayes. The *Nycteria* species group is highlighted in red. Splits with a posterior probability >0.90 are indicated with asterisks. The tree was rooted with Eucoccidiorida and Piroplasmida.

method to determine the occurrence of coinfections within single hosts (16). Coinfections are often undetectable using standard PCR protocols, resulting frequently in the amplification of a single *cytb* sequence and thus to underestimation of Haemosporidian diversity (15). The PCR underestimation in mixed infection cases could, in turn, lead to unreliable morphomolecular parasite identifications and phylogenetic analyses, resulting in erroneous interpretations and conclusions. In the present investigation, using NGS, we have confidently identified the occurrence of two distinct *Nycteria* mt genomes present in mixed infection from *N. grandis* (273VI) and two *Nycteria* monoinfections from *R. alcyone* (289VI) and *M. spasma* (C289). Based on morphological observations of the two *Nycteria* morphotypes and on the large NGS read coverage of the two mt genomes in *N. grandis*, we associated each *Nycteria* species with its corresponding mitochondrial genome. Thus, the mt genome predominantly found in *N. grandis* could consistently be ascribed to *N. medusififormis*, and the minority mt genome could belong to *Nycteria* sp. In the same way, we confidently associated *N. gabonensis* and *N. heischii* with their corresponding mt genomes.

Our phylogenetic analyses support the classification of *Nycteria* parasites as a monophyletic group composed of at least two distinct but closely related parasitic lineages associated with different bat families (12). The distinction between *Nycteria* parasites infecting *Nycteris* and *Rhinolophus* host bats has been proposed previously based on traditional taxonomy (14). Thus, six species have been described and divided into two distinct groups based on the following morphological sexual-stage characteristics in blood (gametocytes) and schizont characteristics: (i) *Nycteris* sp. (*Nycteridae*) parasites with gametocytes arranged as a rosette (*en cocarde*) (*N. medusififormis*, *N. houini*, and *N. erardi*), and (ii) *Rhinolophus* sp. (*Rhinolophidae*) parasites with gametocytes of the dispersed (“diffus”) type (*N. gabonensis*, *Nycteria krampitzi*, and *Nycteria congolensis*) (14). The *N. heischii* parasites from Southeast Asian *M. spasma* were morphologically related to the *Nycteria* from African *Nycteris*, with a nucleus of gametocytes *en cocarde*.

Two phylogenetic placements among all major Haemosporidia parasites and evolutionary scenarios based on multigene phylogenetic analyses (mostly inferred from *cytb* phylogenetic information) have been previously proposed for the *Nycteria* parasite group. *Nycteria* parasites were placed as a sister clade closely related to the mammalian *Plasmodium/Hepaticocystis* parasite clade, suggesting that the origin of mammalian Haemosporidia may have been in bats (11). Alternatively, these parasites were placed closer to *Plasmodium* species from lizards, suggesting an alternative scenario such as a host-switch event between bat and reptile Haemosporidia parasites in their evolutionary history (9). Such a host-switch event between these terrestrial vertebrate host classes has also been assumed for *Polychromophilus* parasites (infecting Minipteridae and Vespertilionidae bats) based on close phylogenetic relationships and shared biological traits (schizogony present in the reticuloendothelial system) (17–20). The mitochondrial genomic phylogenetic analyses presented in this study, using two genetically distantly related Apicomplexan outgroups of Haemosporidia parasites to root phylogenetic reconstructions, did not produce a robust placement of *Nycteria* parasites among the Haemosporidia parasites (21, 22). However, the *Nycteria* parasites that have a hepatic schizogony shared this biological trait with mammalian *Plasmodium* and *Hepaticocystis* parasites (12, 14).

Next-generation sequencing would further open new outlooks into unsuspected and unexplored Haemosporidia parasite diversity, and would help to understand the systematics and evolution of Haemosporidia parasites.

Materials and Methods

Specimens. All animal experiments were approved by the ethical committee of the Museum National d’Histoire Naturelle (Comité Cuvier). A total of 195 bats (six families) were caught using mist nets and harp traps during field expeditions in the Orientale province of the Democratic Republic of Congo (November–December 2013), as well as 90 bats (six families) in Cambodia (July 2005 and January 2006). For morphological parasite identification, thin blood smears were used and blood spots were made on Whatman filter paper to be used later for genome analyses.

One sample each of *N. grandis* (273VI), *R. alcyone* (289VI), and *M. spasma* (C289), infected respectively with *N. medusififormis* and *N. sp.*, *N. gabonensis*, and *N. heischii*, were used for whole mt genome sequencing.

Microscopy. Thin blood smears were fixed with absolute methanol, stained with 10% (vol/vol) Giemsa solution (in phosphate-buffered solution, pH 7.4) for 45 min, and then preserved with mounting medium (Eukitt; Polylabo) and a coverslip. Smears were then carefully screened for morphological examinations with a motorized BX63 upright Olympus microscope at a magnification of 100× and photographed with an Olympus DP72 camera (High-Speed 12.8 Megapixel Image Capture).

DNA Extraction. Whole genomic DNA from *N. grandis*, *R. alcyone*, and *M. spasma* dried blood spots was extracted using a QIAamp DNA Micro Kit (Qiagen) following the manufacturer’s instruction handbook. Samples were eluted in 20 μ L of molecular biology-grade water. Whole genomic DNAs were quantified using a Qubit 2.0 fluorometer (Life Technologies), and concentrations of 0.997 ng/ μ L, 0.74 ng/ μ L, and 0.15 ng/ μ L were respectively obtained for the *N. grandis* (including *N. medusififormis* and *N. sp.* parasites), *R. alcyone* (including *N. gabonensis*), and *M. spasma* samples (including *N. heischii*).

Whole-Genome Sequencing of *Nycteria* Parasites. Whole mitochondrial genome sequencing was performed on *Nycteria* parasites using HiSeq 2000 Illumina paired-read sequencing technology at the Genomics Platform at the Pasteur Institute. The Illumina library preparations were produced using Nextera XT DNA Library Preparation Kits following standard protocols developed by the supplier (Illumina). Briefly, genomic DNA was sheared by nebulization, and sheared fragments were end-repaired and phosphorylated. Blunt-end fragments were A-tailed, and sequencing adapters were ligated to the fragments. Inserts were sized using Agencourt AMPure XP beads (\pm 500 bp; Beckman Coulter Genomics) and enriched using 10 cycles of PCR before library quantification and validation. Hybridization of the Nextera DNA libraries to the flow cell and bridge amplification were performed to generate clusters; paired-end reads of 100 cycles were collected on a HiSeq 2000 (Illumina). Raw sequence files were filtered using the Fqquality tool, read-quality filtering software developed by the gensoft team at the Pasteur Institute, which enables the trimming of the first and last low-quality bases in reads.

Perl software named iSeGWalker was developed to accomplish a de novo genome reconstruction from the reads file in fastq format provided by next-generation sequencing data (Illumina). The exact-matching algorithm removed all variations due to sequencing errors. iSeGWalker was based on a unique and specific seed to Haemosporidia parasites. The sequence TCTTGAGGCAGTTTGTCCCTATCTACC, conserved throughout 72 Haemosporidia species, was chosen as initial seed. The search step is an exact-matching read selection using a regular expression and a very simple Perl script, reading each sequence one by one. Once all matching reads have been selected, a consensus sequence is determined. Once consensus is determined, a new seed, composed by the 30 last consecutive nucleotides, is obtained and a new search is performed. Reads are then mapped using the new sequence as a reference (BWA software) for all samples to identify the parasitic variant present in mixed infection (*N. grandis*).

Assessment of de Novo Reconstruction. The accuracy of the de novo iSeGWalker software was first validated by reconstructing the complete mitochondrial genome of the *Plasmodium falciparum* 3D7 reference strain from NGS data. PCR and nested PCR, using designed specific primers, were also performed to amplify an mt fragment of 1,403 bp from *Nycteria* parasites from *N. grandis* (including rRNA fragments and a portion of the *cox1* sequence). PCR was carried out in a total volume of 25 μ L consisting of 3 μ L DNA, 5 μ L 5× PCR buffer, 1.5 mM MgCl₂, 0.2 mM each dNTP, 1 U Taq DNA polymerase (Promega), and 0.2 mM primers P5 (5′-AACGCCTGABATGRATGGATAA-3′) and P5R (5′-TTCDGGATGWCCAAARAACCAG-3′). PCR cycling conditions were 2 min 30 s denaturation at 95 °C followed by 30 s at 53 °C and 2 min 30 s at 72 °C for 40 cycles, and a final 5-min extension at

72 °C. Nested PCR was performed using 5 µL PCR products (diluted 1/10), 5 µL 5× PCR buffer, 2.5 mM MgCl₂, 0.2 mM each dNTP, 1 U Taq DNA polymerase (Invitrogen), and 0.2 mM primers P4 (5'-TCCATGCTCTCATCGAG-3') and P6iN (5'-ATAATGTCCATCCAGTCCACC-3'). PCR cycling conditions were 2 min 30 s denaturation at 95 °C followed by 30 s at 54 °C and 1 min 30 s at 72 °C for 40 cycles, and a final 5-min extension at 72 °C. The PCR products were sent to Eurofins Genomics for sequencing using nested-PCR primers.

Phylogenetic Analyses. The four complete mitochondrial genomes of *Nycteria* specifically produced for this study were compared with the mitochondrial sequences available in the nucleotide databases for Haemosporidia parasites. Phylogenetic analyses were performed using two different datasets, named 3PCG for the protein alignment of three protein-coding genes (1,014 amino acids; 59 taxa, including 50 Haemosporidians and 9 outgroups) and *cytb* for the DNA alignment of the *cytb* gene (1,054 nt; 110 Haemosporidians). For protein analyses, the Haemosporidian tree was rooted using nine outgroup species belonging to Eucoccidiorida (*Cyclospora cayentanensis*, *Eimeria acervulina*, *Eimeria maxima*, *Eimeria tenella*, and *Hepatozoon catesbianae*) or Piroplasmida (*Babesia bovis*, *Babesia microti*, *Cytauxzoon felis*, and *Theileria parva*). GenBank accession numbers of all the genomic and *cytb* sequences are provided in the trees in Fig. 3 and Fig. S1. Complete mitochondrial genomes were aligned using MUSCLE (23) and

then further adjusted by eye with Se-AL version 2.0a11 (tree.bio.ed.ac.uk/software/seal/). For *cytb* datasets, the best-fitting model of sequence evolution (GTR+I+G) was selected under jModelTest 2.1.4 (24) using the Akaike information criterion. Phylogenetic relationships were inferred using Bayesian analyses with MrBayes version 3.2.1 (25), and posterior probabilities were calculated using four independent Markov chains run for 10 million Metropolis-coupled Markov chain Monte Carlo generations, with tree sampling every 1,000 generations and a burn-in of 25%. For the protein alignment (3PCG dataset), phylogenetic relationships were reconstructed with the same Bayesian approach by applying the mixed model (25).

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