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New member of *Plasmodium* (*Vinckeia*) and *Plasmodium cyclopsi* discovered in bats in Sierra Leone – nuclear sequence and complete mitochondrial genome analyses ☆

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ABSTRACT

Malaria remains the most important arthropod-borne infectious disease globally. The causative agent, *Plasmodium*, is a unicellular eukaryote that develops inside red blood cells. Identifying new *Plasmodium* parasite species that infect mammalian hosts can shed light on the complex evolution and diversity of malaria parasites. Bats feature a high diversity of microorganisms including seven separate genera of malarial parasites. Three species of *Plasmodium* have been reported so far, for which scarce reports exist. Here we present data from an investigation of *Plasmodium* infections in bats in the western Guinean lowland forest in Sierra Leone. We discovered a new *Plasmodium* parasite in the horseshoe bat *Rhinolophus landeri*. *Plasmodium cyclopsi* infections in a member of leaf-nosed bats, *Doryrhina cyclops*, exhibited a high prevalence of 100%. Phylogenetic analysis of complete mitochondrial genomes and nine nuclear markers recovered a close relationship between *P. cyclopsi* and the new *Plasmodium* parasite with the rodent species *Plasmodium berghei*, a widely used *in vivo* model to study malaria in humans. The data suggests that the "rodent/bat" *Plasmodium* (*Vinckeia)* clade represents a diverse group of malarial parasites that would likely expand with a systematic sampling of small mammals in tropical Africa. Identifying the bat *Plasmodium* repertoire is central to our understanding of the evolution of *Plasmodium* parasites in mammals.

1. Introduction

Bats (Chiroptera) are a highly diverse group of mammals, second only to rodents. They are distributed almost worldwide and feature diverse life history traits (https://batnames.org/home.html). Bats provide valuable ecosystem services as insect predators, pollinators, seed dispersers, and nutrient recyclers (Kalka et al., 2008; Ramírez-Fráncel et al., 2022). Their exceptional immune systems and relatively long lifespans correlate with tolerating a wide range of intracellular microorganisms without displaying symptoms (Brook and Dobson, 2015). Bats are natural

☆ **Note:** Nucleotide sequence data reported in this paper are available in GenBank™ under the accession numbers **OR671203** (for NW2664), **OR671204** (for NW2690) (*Plasmodium* mt genome sequences), **OR888012 – OR888029** (*Plasmodium* nuclear gene sequences) and **OR888030 – OR888035** (sequences of bat host genotyping).

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hosts to *Plasmodium* and related species that belong to a diverse group of haemosporidian blood parasites (Phylum Apicomplexa). The order Haemosporida also includes the *Plasmodium* spp. that cause malaria in humans (Garnham, 1966; Martinsen et al., 2013; Pacheco and Escalante, 2023). The bat-infecting haemosporidian parasites comprise species classified into seven genera including *Plasmodium, Hepatocystis, Nycteria, Polychromophilus* (including *Bioccala, Biguetiella*), *Dionisia, Johnsprentia,* and *Sprattiella.* Together, they account for approximately one-third of all formally described haemosporidian species that infect mammals (Pacheco and Escalante, 2023). Their distribution covers a broad global range including Europe, Africa, southeastern Asia, Australia, and North and South America (Perkins and Schaer, 2016). Surprisingly, despite their high diversity and wide distribution, little is known about the chiropteran haemosporidian biology and prevalence, especially the *Plasmodium* spp. It has been proposed that bats are the ancestral hosts of some genera of haemosporidian parasites currently found in rodents and primates, which underscores the importance of bat hosts in the study of parasite evolution (Killick-Kendrick and Peters, 1978; Schaer et al., 2013; Lutz et al., 2016; Galen et al., 2018).

Murine *Plasmodium* spp. have advanced malaria research since their first discovery in the 1940s, when an African mosquito species, *Anopheles dureni,* and the arboreal woodland ticket rat (*Grammomys dolichurus*) were identified as natural hosts of the first type strain of *Plasmodium (Vinckeia) berghei* (Vincke, 1946; Vincke and Lips, 1948). The successful transfer of *Plasmodium berghei* into white mice marked the beginning of rodent malaria models; different strains of *P. berghei* (Vincke and Lips, 1948), and three additional *Plasmodium* spp., *Plasmodium vinckei* (Rodhain, 1952), *Plasmodium yoelii* (Landau and Chabaud, 1965; Killick-Kendrick, 1974), and *Plasmodium chabaudi* (Landau, 1965) were isolated from wild rodents and adapted to laboratory mice. Murine *Plasmodium* infections are widely used as *in vivo* models to study malaria in humans (Carlton et al., 2001; De Niz and Heussler, 2018). The four rodent *Plasmodium* spp. and their respective laboratory strains differ in various aspects including virulence in mice and the natural host (Killick-Kendrick and Peters, 1978; Wykes and Good, 2009; Conteh et al., 2020).

The rodent *Plasmodium* taxa were traditionally classified in the *Plasmodium* subgenus *Vinckeia* together with *Plasmodium* spp. of other mammals including bats, mainly based on the parasite morphology (Garnham, 1966). However, emerging molecular evidence indicates that this subgenus is not a monophyletic group (Pacheco et al., 2011; Martinsen et al., 2016; Templeton et al., 2016; Pacheco et al., 2022). Still, a chiropteran/rodent *Plasmodium* clade has been recovered in phylogenetic analyses (Schaer et al., 2013; Galen et al., 2018). To date, bat *Plasmodium* (*Vinckeia*) parasites only comprise three species, namely *Plasmodium voltaicum*, originally found in the Angolan fruit bat (*Myonycteris angolensis*) in Ghana (van der Kaay, 1964), *Plasmodium rousetti*, described from the Egyptian rousette (*Rousettus aegyptiacus*) in the Democratic Republic of the Congo (Riel et al., 1951), and *Plasmodium cyclopsi* described from the hipposiderid cyclops roundleaf bat (*Doryrhina cyclops*) in Gabon (Landau and Chabaud, 1978). Since their discovery, the chiropteran *Plasmodium* spp. have been classified as related to the rodent *Plasmodium* spp. based on differential morphological characteristics (Garnham, 1966; Landau and Chabaud, 1978). Only two bat *Plasmodium* spp., *P. cyclopsi* and *P. voltaicum,* have been rediscovered since their first description and display exceptionally tight links with the two rodent *Plasmodium* spp., *P. berghei* and *P. yoelii,* respectively, indicative of multiple switches between bat and rodent hosts (Schaer et al., 2013).

In this study we present data from an investigation of *Plasmodium* infections in bats in the Gola Rainforest National Park (GRNP), Sierra Leone. *Plasmodium cyclopsi* infections were detected in *D. cyclops* bats, and we discovered a putative new *Plasmodium* sp. in Lander's horseshoe bat (*Rhinolophus landeri*). We generated complete mitochondrial genomes (mtDNA) and a dataset of multiple nuclear markers of the two parasite taxa to explore the phylogenetic relationships of bat *Plasmodium* taxa among the group of mammalian *Plasmodium* spp.

2. Materials and methods

2.1. Ethics

Sampling permission was granted by the GRNP, and permits were authorized by the Ministry of Agriculture and Forestry, Freetown, 00232, Sierra Leone. All techniques employed for capture and preservation complied with standard methods for measuring and monitoring mammal diversity and guidelines approved by the American Society of Mammalogists (Sikes et al., 2016).

2.2. Study site, field sampling and microscopy

We performed an ad hoc survey of bats in the GRNP at the boundary with Liberia (Supplementary Fig. S1), focusing on the detection and thorough molecular characterization of *Plasmodium* parasite infections in the bat species *D. cyclops.* It was carried out as part of a study investigating the diversity and distribution of bats in the GRNP. A total of 120 bat individuals belonging to five bat families (Hipposideridae, Rhinolophidae, Nycteridae, Pteropodidae and Vespertilionidae) were recorded in the survey (Supplementary Table S1). Mist nets were used to capture bats across potential flyways, while individuals of the species *D. cyclops* were captured close to their roosting sites in cavities of hollow trees. Bats were removed from the nets and temporarily kept solitary in cotton bat bags. For bat species identification, different identification keys were used, e.g., Rosevear (1965) and Meester and Setzer (1971). Wing punches were collected for each bat individual, while blood was only sampled from a subset of bats (Supplementary Table S2). Blood sampling followed approved animal care and protocols (Sikes et al., 2016). Blood was obtained either from the venous puncture of the vein running along the anterior edge of the propatagium or antebrachial membrane, or venous puncture of the major vein in the interfemoral membrane following Kurta and Kunz (1988). A small amount of blood was collected with a heparinized capillary tube, and it was ensured that the bleeding had stopped before releasing the animal. Immediately after blood collection the sample was used to prepare a thin blood smear and to collect blood dots on DNA FTA cards (GE Healthcare) for morphological and molecular identification of the blood parasites. Thin blood smears were prepared in the field and fixed in 100% methanol for approximately 30 s. All blood smears were stained with Giemsa to differentiate blood cells and monitor blood cell infections with light microscopy at a magnification of $\times$1,000 with immersion oil. The parasitemia (as the percentage of infected erythrocytes) was recorded for each infected bat. An average number of erythrocytes per field was calculated by counting the total number of erythrocytes observed in two to three fields and dividing by two or three, respectively. Then, the number of parasites was counted across 40–50 fields of the slide, thereby choosing fields of comparable erythrocyte density. Parasitemia is given as the number of parasites per total estimated erythrocyte number (approximately 8,000–15,000 erythrocytes). Samples with detected parasites were examined for an extended period to investigate the morphology of the parasitic blood stages which were compared with original species descriptions. Pictures of parasites were taken with a digital camera and software (Olympus SC50 Olympus Soft Imaging Solutions GmbH, Munster, Germany).

All bats were released at the capture sites except two individuals that were anesthetized with isoflurane and subsequently deposited as voucher specimens in the mammal collections of the Museum für Naturkunde, Berlin (Catalogue numbers ZMB_Mam_105661-105662) for subsequent studies (Supplementary Table S2).

2.3. Molecular methods

A subset comprising individuals of the bat species *D. cyclops* (bat family Hipposideridae) and *Rhinolophus landeri* (Rhinolophidae), captured at four and two different sampling sites, respectively, were screened for infections with haemosporidian parasites (Supplementary Table S2). While the focus was on *Plasmodium* parasite infections in *D. cyclops, R. landeri* individuals were included in the subset as ambiguous haemosporidian cytochrome *b* sequences were discovered in one *R. landeri* sample, that was originally found to be infected with *Nycteria* parasites (Schaer et al., 2015).

For molecular analyses of the parasites, whole genomic DNA was extracted from dried blood dots on DNA FTA classic cards using the DNeasy extraction kit (QIAGEN® GmbH, Hilden, Germany), following the manufacturer's protocol for animal tissues with minor modifications and elution of samples in 80 µl of AE buffer (10 mM Tris–Cl, 0.5 mM EDTA; pH 9.0). The initial PCR screening of the samples was performed using the AllTaq Master Mix Kit (QIAGEN® GmbH, Hilden, Germany) with 2–4 µl of genomic DNA as the template and targeting partial sequences of the mitochondrial cytochrome *b* (*cytb*) and the apicoplast caseinolytic protease (*clpC*). For samples with verified haemosporidian infections, a fragment (550 nucleotides (nt)) of the nuclear elongation factor 2 (*ef2*) of the parasite was targeted (Schaer et al., 2013). All primers are listed in Supplementary Table S3.

Two samples (sample nos. NW2664 and NW2690) were selected for subsequent amplification and sequencing of complete mtDNA genomes and additional nuclear genes of the parasites to genetically characterize the taxa in more detail. Selective whole genome amplification (WGA) was performed on the genomic DNA using the REPLI-g Mini Kit (QIAGEN® GmbH, Hilden, Germany). The WGA-DNA sample of the co-infected sample NW2664 (co-infected with *Plasmodium* and *Nycteria*) was dominated by *Plasmodium* DNA; all electropherograms were carefully inspected and all recovered sequences could be assigned to *Plasmodium* (all individual sequences/raw reads were compared with published reference sequences in NCBI BLASTn).

Different established molecular markers of the bat hosts were sequenced for one representative *D. cyclops* (sample no. NW2651) and one *R. landeri* sample (no. NW2264) to verify morphological identification of the bat species. The mitochondrial NADH dehydrogenase 1 (*ND1*), the nuclear Recombination activating gene 1 (*rag1*), the nuclear introns Acyl-CoA oxidase 2, intron 3 (*acox2*), and ROGDI-like protein, intron 7 (*rogdi*) were amplified, sequenced, and compared with published reference sequences in NCBI GenBank, confirming the morphological identification of these bat host species (Supplementary Table S4, accession numbers OR888030 – OR888035).

2.3.1. Complete mitochondrial genomes

Complete mtDNA genomes were amplified for the two selected parasite samples using the TaKaRa LA PCR Kit (Takara Bio Europe SAS) (Pacheco et al., 2011, 2022). The mtDNA genomes were amplified using a nested PCR approach with the outer oligonucleotides AE170/AE171 and the nested oligonucleotides AE176/AE136 (Supplementary Table S3). The following amplification conditions were used: partial denaturation at 94 °C for 1 min, followed by 30 cycles with 30 s at 94 °C, and 7 min at 67 °C. The final extension was carried out for 10 min at 72 °C. While exposing the agarose gel to UV light for as short a time as possible, PCR products of each sample (approximately 6 kb) were excised from the gel and purified with the QIAquick Gel Extraction Kit according to the manufacturers protocol (QIAGEN® GmbH, Hilden, Germany). Several independent PCR products were purified per sample. Published and newly designed primers were selected and combined to ensure that sequences partially overlapped and covered the full length of the mtDNA genome (Supplementary Table S3). To avoid assembling chimeras of mixed haplotypes, two to four sequences for most regions were generated from independent PCR products to verify single *Plasmodium* haplotype infections.

All mtDNA sequences were manually edited and individual sequence assemblies per sample were generated. The complete mtDNA genome sequences of the two *Plasmodium* lineages from bats from Sierra Leone were aligned with published haemosporidian mtDNA genome sequences available from GenBank. The alignment was constructed with a total of 77 sequences using ClustalX v2.0.12 and MUSCLE as implemented in SeaView v4.3.5 (Gouy et al., 2010) with manual editing. Phylogenetic relationships were estimated using a Bayesian analysis implemented in MrBayes v3.2.7 with the default priors (Ronquist and Huelsenbeck, 2003) and a maximum likelihood (ML) method using IQ-TREE (Nguyen et al., 2015). The phylogenetic relationships were inferred on this alignment using six partitions (Pacheco et al., 2018) corresponding to the three non-protein-coding regions between the open reading frames (ORFs; fragmented ssrRNA and lsrRNA) and the three protein-coding genes, keeping their order in the mtDNA genome. In the case of Bayesian analysis, a general time-reversible model with gamma-distributed substitution rates and a proportion of invariant sites (GTR + Γ + I) was used for each partition (model with the lowest Bayesian Information Criterion (BIC) scores for the alignments and each partition as estimated by MEGA v7.0.14 (Kumar et al., 2016)). Bayesian support was inferred for the nodes in MrBayes by sampling every 1000 generations from two independent chains lasting 4×10^6 Markov chain Monte Carlo steps. The chains were assumed to have converged once the value of the potential scale reduction factor was between 1.00 and 1.02, and the average standard deviation of the posterior probability was <0.01. Once convergence was reached, 25% of the samples were discarded as 'burn-in'. GenBank accession numbers of all sequences used in this analysis are shown in the phylogenetic tree and are given in an alignment file (available as a raw data file in Mendeley Data repository https://doi.org/10.17632/w3w3rrdcrv.1). In the Maximum Likelihood (ML) analysis, GTR + F + I + G4 was the substitution model obtained with ModelFinder (Kalyaanamoorthy et al., 2017). The best tree is the one with the highest likelihood score among all evaluated tree topologies. Support values were generated through Ultrafast bootstrap approximation (UFBoot) (Minh et al., 2013) with 1,000 replicates. For both phylogenetic analyses, *Plasmodium* spp. infecting ungulate species were selected as the outgroup.

2.3.1.1. Partial mitochondrial sequence analysis. No complete mtDNA genomes are available for the references of *P. cyclopsi* from Liberia (*P. cyclopsi* L-4-1) and *P. voltaicum* from Guinea (*P. voltaicum* G-1-1). Therefore, phylogenetic analyses using the concatenated (available) partial sequences of *cox1* and *cytb* genes of the two references were carried out to evaluate their phylogenetic placement in relation to the *P. cyclopsi* and *Plasmodium* sp. samples of the study. The concatenated alignment featured a length of 1,800 nt without gaps (744 nt *cox1*, 1056 nt *cytb*). Bayesian analysis was conducted with five million generations (partitions and models given in Supplementary Table S5) and ML analysis used the substitution model TIM3 + I + Gamma and a nodal support of 1,000 thorough bootstraps.

Further, the phylogenetic relationships with *Plasmodium* (*Vinckeia*) infections in wild rodents in Gabon were explored. The available partial *cytb* sequences (Boundenga et al., 2019) were aligned with those of all bat *Plasmodium* lineages together with other reference sequences of different rodent *Plasmodium* spp. ML analysis of the 696 nt long *cytb* alignment was carried out using the substitution model GTR + G and nodal support was evaluated using 10,000 replicates (thorough bootstrap).

2.3.2. Nuclear datasets

A total of nine nuclear markers were amplified following (Schaer et al., 2013). PCRs were carried out using the ExTaq Polymerase kit (TaKaRa®, Europe) and PCR products were Sanger-sequenced with the amplification primers. All primers are listed in Supplementary Table S3. All DNA sequences were assembled and manually edited in the software Geneious Prime 2022.1 (https://www.geneious.com). Gene sequences were aligned with reference sequences of the different rodent *Plasmodium* spp. and their diverse strains and the taxa *Plasmodium vivax*, *Plasmodium knowlesi* and *Plasmodium falciparum* using the MAFFT algorithm (Katoh and Standley, 2013; Katoh et al., 2002). All individual gene alignments were concatenated. The concatenated nuclear dataset of 22 sequences included a total of 4,488 nt (excluding gaps) of nine genes; 516 nt of the actin1 (*act1*), 489 nt of actin2 (*act2*), 210 nt of adenylosuccinate lyase (*asl*), 621 nt of cysteine proteinase (*cyspro*), 597 nt of dihydrofolate reductase/thymidylate synthase (*dhfr/ts*), 579 nt of the elongation factor 2 (*ef2*), 375 nt of histone H2A (*h2a*), 477 nt of ookinete surface protein (*p25*), and 624 nt of poly-ubiquitin (*ubi*). Accession numbers are listed in Supplementary Table S6. Phylogenetic relationships were evaluated by using Bayesian inference and ML methods. Data were divided into partitions according to genes and first, second and third codon positions of the protein-coding genes were defined.

For the Bayesian analysis, different DNA substitution models and partition schemes were tested in PartitionFinder v.2 (Lanfear et al., 2014) and the best partition schemes and models were used (see Supplementary Table S5). Bayesian inference was conducted in MrBayes 3.2.7 (Ronquist and Huelsenbeck, 2003) via the CIPRES Science Gateway Web Portal V3.3 (https://doi.org/10.1109/GCE.2010.5676129) with two runs of four chains (three heated, one cold, temperature = 0.1) each for five million generations. Bayesian support was inferred as in the mtDNA genome Bayesian analysis (e.g., sampling every 1,000 generations, convergence of runs once the value of potential scale reduction factor was between 1.00 and 1.02, and the average standard deviation of the posterior probability was <0.01). Effective sample size (ESS) was greater than 1,000. The first 25% of trees were discarded as "burn-in". ML analysis was conducted in raxmlGUI v. 2.0.1 (Edler et al., 2021) and the software ModelTest-NG 0.1.7 (Darriba et al., 2020) was used to test different DNA substitution models. The concatenated nuclear dataset was carried out using the substitution model GTR + I (proportion of invariant) + Gamma (rate heterogeneity) and the nodal support was evaluated using 1,000 thorough bootstrap pseudoreplicates (Stamatakis et al., 2008). For both phylogenetic analyses, *P. falciparum* was selected as the outgroup.

A second analysis of a concatenated dataset of eight nuclear genes was carried out for the 22 sequences plus the reference sequences from *P. cyclopsi* from Liberia (*P. cyclopsi* L-4-1) and *P. voltaicum* from Guinea (*P. voltaicum* G-1-1) (accession numbers listed in Supplementary Table S6). The individual gene alignments were cut to the length of the two reference sequences. Data for only seven and three out of the eight genes were available for *P. cyclopsi* from Guinea and *P. voltaicum,* respectively, resulting in overall missing data of 3.1% in the concatenated dataset, 17.9% for *P. cyclopsi* (L-4-1) and 57.5% for *P. voltaicum.* The concatenated nuclear dataset of 24 sequences included a total of 3,337 nt excluding gaps of eight genes (417 nt *actin-1*, 441 nt *actin-2*, 204 nt *asl*, 621 nt *cyspro*, 594 nt *dhfr/ts*, 513 nt *ef2*, 357 nt *h2a*, and 189 nt *ubi*). Bayesian analysis was carried out analogous to the first concatenated nuclear dataset (partitions and models given in Supplementary Table S5). ML analysis was carried out using the substitution model TIM3 + I + Gamma and the nodal support was evaluated using 500 thorough bootstrap pseudoreplicates (Stamatakis et al., 2008). Third and fourth analyses of concatenated nuclear datasets were carried out to evaluate the phylogenetic placement of the reference sequences from *P. cyclopsi* from Liberia (*P. cyclopsi* L-4-1) and *P. voltaicum* from Guinea (*P. voltaicum* G-1-1) without missing data. Therefore, one ML analysis of a concatenated dataset was conducted for the available sequences of seven nuclear genes for *P. cyclopsi* L-4-1 (2,743 nt length excluding gaps: 417 nt *actin-1*, 441 nt *actin-2*, 204 nt *asl*, 621 nt *cyspro*, 513 nt *ef2*, 357 nt *h2a*, and 189 nt *ubi*) and another ML analysis for the sequences of three nuclear genes for *P. voltaicum* (1,420 nt length excluding gaps: 204 nt *asl*, 615 nt *cyspro*, and 594 nt *dhfr/ts*), both with the substitution model TIM3 + I + Gamma and the nodal support of 1,000 thorough bootstraps. All resulting phylogenetic trees were displayed in FigTree v1.4.4 (https://github.com/rambaut/figtree/).

2.3.3. Estimates of evolutionary divergence

Analyses of evolutionary divergence estimates between mtDNA genome sequences, concatenated nuclear and partial mitochondrial and individual gene sequences from rodent and bat *Plasmodium* spp. were conducted using the Kimura 2-parameter model (Kimura, 1980). The number of base substitutions per site between the sequences and standard error estimates were calculated (Supplementary Tables S7–S10). The rate variation among sites was modeled with a gamma distribution (shape parameter = 4). Codon positions included were 1st + 2nd + 3rd. The evolutionary analyses were carried out in MEGA 7 (Kumar et al., 2016).

2.3.4. Data accessibility

All sequence data generated by this study have been deposited in GenBank (accession numbers: **OR671203-OR671204**, **OR888012-OR888029**, **OR888030-OR888035**). The electronic supplementary file accompanying this article contains additional details of samples used in this study and models of sequence evolution used in phylogenetic analyses. Sequence alignments and genetic distance tables have been deposited in the Mendeley Data repository (Mendeley Data, V1, https://doi.org/10.17632/w3w3r-rdcrv.1).

3. Results

3.1. Identification of Plasmodium spp. in candidate bat host taxa

Plasmodium parasites were detected in all *D. cyclops* individuals (10/10, 100%; four different sampling sites) and in one individual of *R. landeri* (1/2, 50%) using morphological (Fig. 1) and molecular screening. PCR and sequencing of a partial sequence of the parasites´ *ef2* gene (∼550 bp) was performed to verify infection status and confirm identification of *Plasmodium* parasites. The *Plasmodium ef2* nucleotide sequences of all 10 *D. cyclops* individuals were 100% identical to each other and to the published sequences of *P. cyclopsi,* thus representing a single haplotype (GenBank accession numbers: KF159728, KF159729; (Schaer et al., 2013)). The *Plasmodium ef2* sequence of *R. landeri* (NW2664) featured a 99.1% identity (five bases differed) with *P. cyclopsi,* pointing to a distinct lineage. Infections with the haemosporidian taxon *Nycteria* were verified in the two *R. landeri* individuals (Schaer et al., 2015) (Supplementary Table S2).

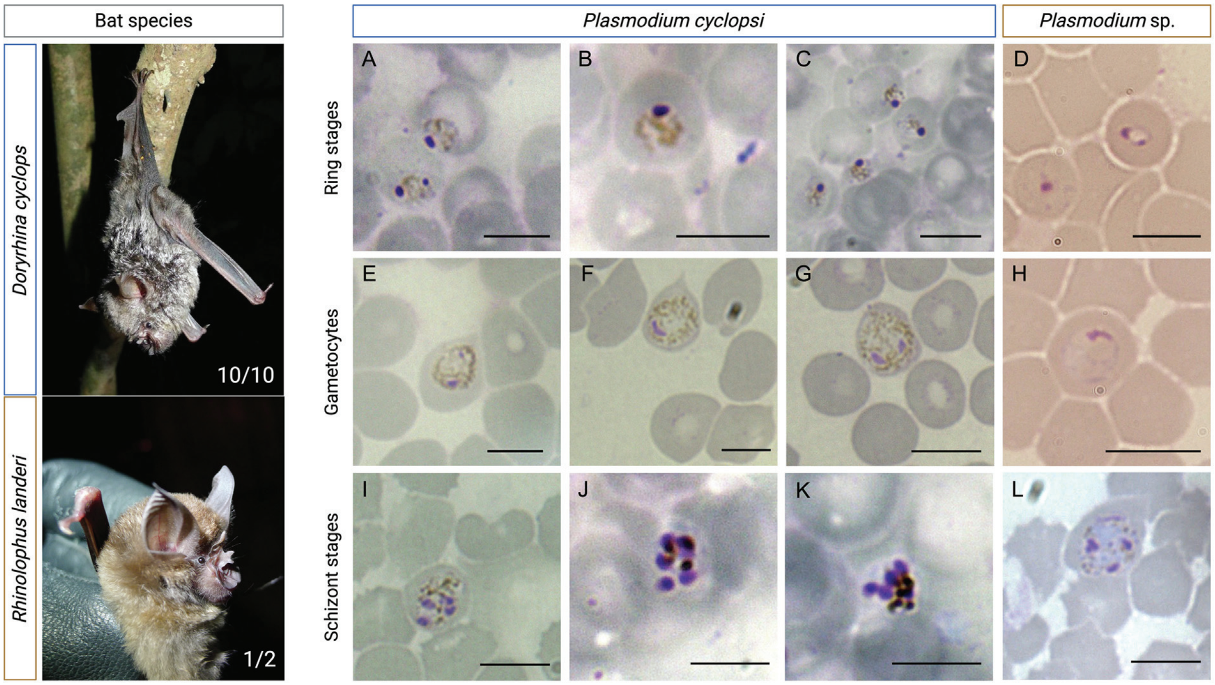

Fig. 1. Representative micrographs of Giemsa-stained blood films of *Plasmodium*-infected bat species *Doryrhina cyclops* and *Rhinolophus landeri.* Photographs show the bat host species *D. cyclops* and *R. landeri*, and the respective prevalence of *Plasmodium* infections (number of infected/number of investigated individuals). Micrographs (A–L) show early blood stages (ring), gametocyte and schizont stages of *Plasmodium cyclopsi* of *D. cyclops*and of *Plasmodium* sp. of *R. landeri* (NW2664). Note the large and dark staining nuclei in ring stages (A–D) and double gametocyte infection in G. In *P. cyclopsi* schizonts, three to five nuclei are hallmarks (I–K); schizonts of *Plasmodium* sp. featured three to four nuclei (L and Supplementary Fig. S2), Bars = 5 µm.

3.2. Morphology of Plasmodium blood stages

Morphology of the parasite blood stages detected in *D. cyclops* resembled both the original descriptions of the species *P. cyclopsi* from Gabon (Landau and Chabaud, 1978) and the morphology of *P. cyclopsi* reported recently from Liberia (Schaer et al., 2013). The early blood stages, thin and fragile ring forms, feature a vacuole limited by a narrow border of cytoplasm and a relatively large nucleus (Fig. 1A–C). The developing and mature gametocyte stages exhibited less dense nuclei than the rings with hemozoin evenly distributed in the cytoplasm (Fig. 1E–G). The erythrocytic schizonts are very compact, with small dense nuclei (stained in purple) and a mass of granules that occasionally contain a large grain of black pigment. Usually, three to five nuclei were visible in the schizonts (Fig. 1I–K). The 10 individuals of *D. cyclops* from Sierra Leone exhibited a mean parasitemia of 0.17% (<0.001–0.3%) (Supplementary Table S2).

Systematic microscopic screening of the *R. landeri* sample (NW2664), which displayed *Nycteria congolensis* infections (Schaer et al., 2015), recovered concurrent low infections with candidate *Plasmodium* parasite stages (Fig. 1D, H, L and Supplementary Fig. S2). The *Plasmodium* sp. ring stages from *R. landeri* were thin, with smaller and less dense nuclei in comparison with *P. cyclopsi* (Fig. 1D and Supplementary Fig. S2A, C). The developing gametocyte features a large, elongated nucleus, a prominent vacuole, and absence of hemozoin aggregation (Fig. 1H). Schizont stages featured three to four nuclei, and again hemozoin was barely detectable (Fig, 1L and Supplementary Fig. S2B, D–F). The presence of schizonts unequivocally identifies the parasites as species of *Plasmodium,* since all other haemosporidian parasite taxa lack erythrocytic schizogony and instead progress from pre-erythrocytic schizogony to gametocyte maturation in infected erythrocytes (Galen et al., 2018).

3.3. Phylogenetic relationships of bat Plasmodium taxa

We performed *de novo* assemblies of the mitochondrial genomes of the two bat *Plasmodium* taxa (*P. cyclopsi*, representative sample NW2690 and *Plasmodium* sp., sample NW2664). The resulting complete mtDNA genome sizes were 5,956 bp for *Plasmodium* sp. (OR671203) and 5,957 bp for *P. cyclopsi* (OR671204). As expected for *Plasmodium* spp. (Feagin et al., 2012), the mtDNA genomes encompass the three protein-coding genes *cytb*, *cox1* and *cox3* and several highly fragmented rRNAs. The Bayesian and ML analyses of complete mtDNA genomes recovered the bat *Plasmodium* parasites *P. cyclopsi* and *Plasmodium* sp. as two separate lineages that are closely related to *P. berghei* (Fig. 2A and Supplementary Fig. S3). The bat *Plasmodium* parasites and the *P. berghei* strains form a sister clade to the species *P. yoelii*, while the rodent species *Plasmodium atheruri*, *P. vinckei* and *P. chabaudi* are more distantly related (Fig. 2A and Supplementary Fig. S3).

We next performed phylogenetic analysis of the concatenated dataset of nine nuclear gene sequences of the two bat *Plasmodium* taxa and the rodent *Plasmodium* parasites. In perfect agreement with the mtDNA genome phylogenetic analysis, a close relationship of *P. cyclopsi* and the *Plasmodium* sp. lineage (from *R. landeri*) with the rodent-infecting species *P. berghei* was recovered with high support and the taxa appear to share a recent common ancestor (Fig. 2B). The clade of *P. yoelii* strains, another rodent-infecting species, groups as a sister clade more distantly related to the "bat *Plasmodium*/*P. berghei*" group (Fig. 2B).

Additional analyses were carried out to evaluate the relationships between the *Plasmodium* taxa of this study with the previously published bat *Plasmodium* taxa (Schaer et al., 2013). A concatenated analysis of partial mitochondrial gene sequences included data from one *P. cyclopsi* sample from *D. cyclops* in Liberia and the bat *Plasmodium* sp. *P. voltaicum* from *M. angolensis* in

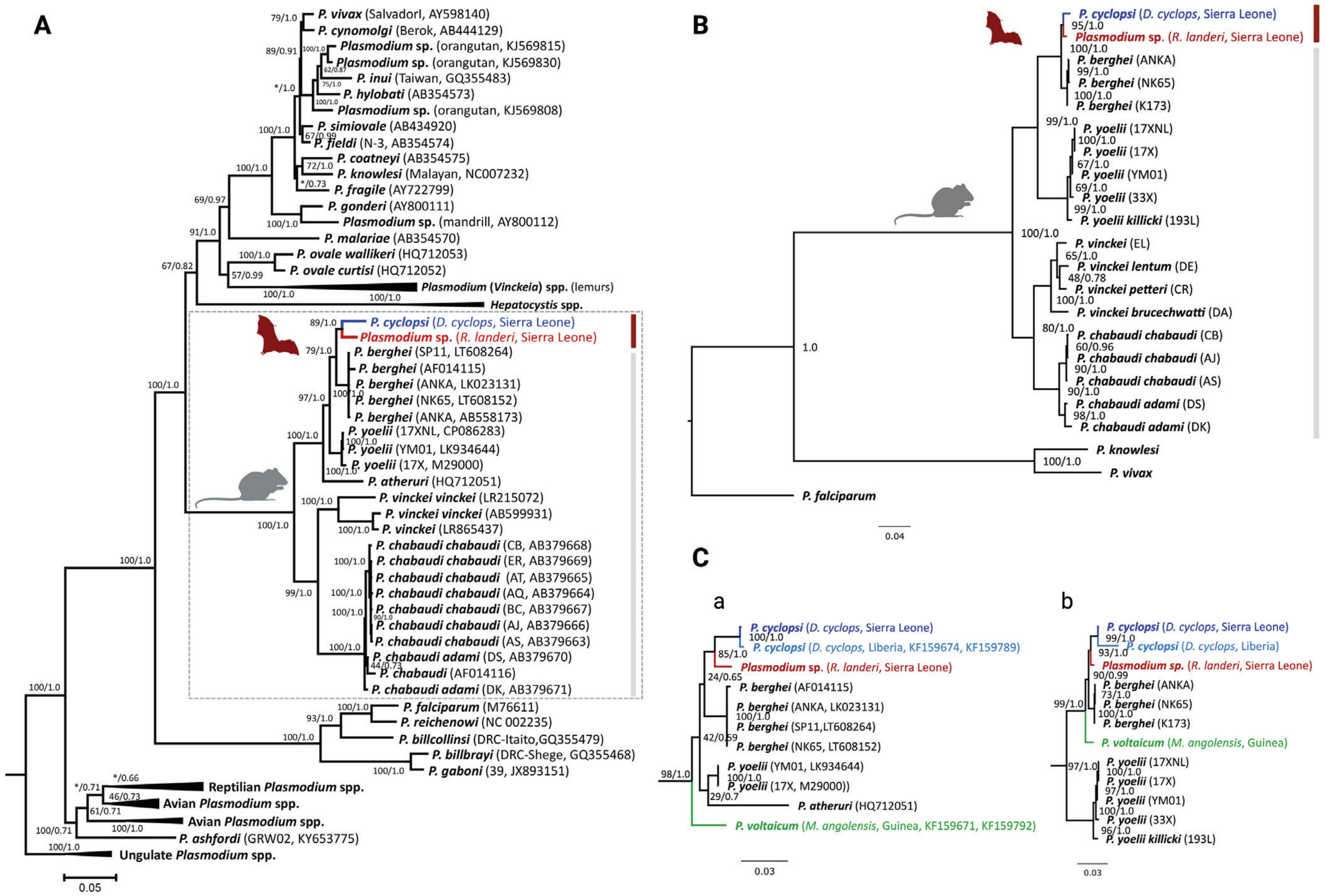

Fig. 2. Phylogenetic relationships of bat *Plasmodium* taxa. Bootstrap/posterior probabilities are given and the asterisk indicates inconsistency between phylogenetic methods. (A) mtDNA genome phylogeny of *Plasmodium* parasites in the context of the major *Plasmodium* clades of mammals, birds and squamates. The dataset included 77 mtDNA sequences and a total of 5,268 nucleotides (nt) without gaps. *Plasmodium* spp. infecting ungulates were used as an outgroup. (B) Phylogeny of rodent and bat *Plasmodium* parasites using a concatenated dataset of nine nuclear genes. The dataset included a total of 4,488 nt (excluding gaps) of nine genes. The taxon *Plasmodium falciparum* was used as an outgroup. (C) Phylogenetic relationships of bat and rodent *Plasmodium* parasites. This analysis included previously published bat *Plasmodium* sequences of *P. cyclopsi* and *Plasmodium voltaicum*. Phylogenetic analyses of the concatenated partial sequences of *cox1* and *cytb* (1,800 nt) (a) and the concatenated nuclear dataset of eight genes (3,337 nt) (b). *Plasmodium falciparum* was used as outgroup. NCBI accession numbers are given in Supplementary Table S6. Full trees are given in Supplementary Figs. S7.

Guinea. The two *P. cyclopsi* sequences group in one clade with high support (100/1.0) and closely to *Plasmodium* sp. (*R. landeri*). Of note, the analysis recovered *P. voltaicum* as a basal clade outside the other bat *Plasmodium* taxa and outside the rodent taxa *P. berghei*, *P. yoelii* and *P. atheruri* (Fig. 2C). In contrast, the analysis of the concatenated dataset of eight nuclear genes recovered *P. voltaicum* basal to the *P. cyclopsi*/*Plasmodium* sp./*P. berghei* clade (Fig. 2C). The analysis comprised a large amount of missing data for *P. voltaicum* (only three out of eight genes were available); a subsequent analysis without missing data (using three genes) confirmed this topology (Supplementary Fig. S4).

To explore the phylogenetic relationships of the bat *Plasmodium* spp. in the context of rodent *Plasmodium* taxa that have been recently discovered in wild rodent species in Gabon (Boundenga et al., 2019), a phylogenetic analysis of the partial *cytb* gene was carried out and confirmed the close relationship of *P. cyclopsi* and *Plasmodium* sp. with *P. berghei.* In agreement with the other partial mitochondrial gene analysis, *P. voltaicum* was recovered outside of the other bat *Plasmodium* taxa and *P. berghei*, *P. yoelii* and *P. atheruri* (Supplementary Fig. S5).

3.3.1. Genetic divergences between bat and rodent Plasmodium taxa

The estimates of the genetic divergences between the complete mtDNA genomes of the closely related species *P. yoelii, P. berghei, P. cyclopsi,* and *Plasmodium* sp. were between 0.028 and 0.033 (Fig. 3 and Supplementary Tables S7, S11). Accordingly, comparison of the three protein-coding genes of the mtDNA follow the same pattern (Supplementary Table S7). The alignment comparison of the translated nucleotide sequences of *cytb*, *cox1* and *cox3 genes* of *P. cyclopsi*, *P. berghei* and *Plasmodium* sp. revealed few non-synonymous mutations/different amino acids per gene (Supplementary Table S12). The estimates of the genetic divergences between the nuclear gene sequences differ more and were between 0.013 and 0.053 (Fig. 3, Supplementary Tables S8, S11).

Comparisons between *P. berghei* (ANKA strain), *P. cyclopsi*, and *Plasmodium* sp. (ex *R. landeri*) reveal similar divergence for the mtDNA genomes (0.027–0.033), but lower values in the nuclear sequences (0.011–0.017) (Fig. 3, Supplementary Tables S7–S11). As expected, comparing the estimates of genetic divergences between the *P. cyclopsi* sample of this study and one *P. cyclopsi* from Liberia using the partial mitochondrial gene sequences resulted in a low divergence of 0.003 (Supplementary Table S9). Of note, the nuclear genetic divergence of 0.019 implies the possibility that the two parasites could belong to two different strains. Based on the limited available data, *P. voltaicum* appears to represent a distinct species with genetic divergences of over 0.02 compared with each of the closely related rodent *Plasmodium* spp., *P. berghei*, *P. yoelii and P. atheruri,* and the other bat

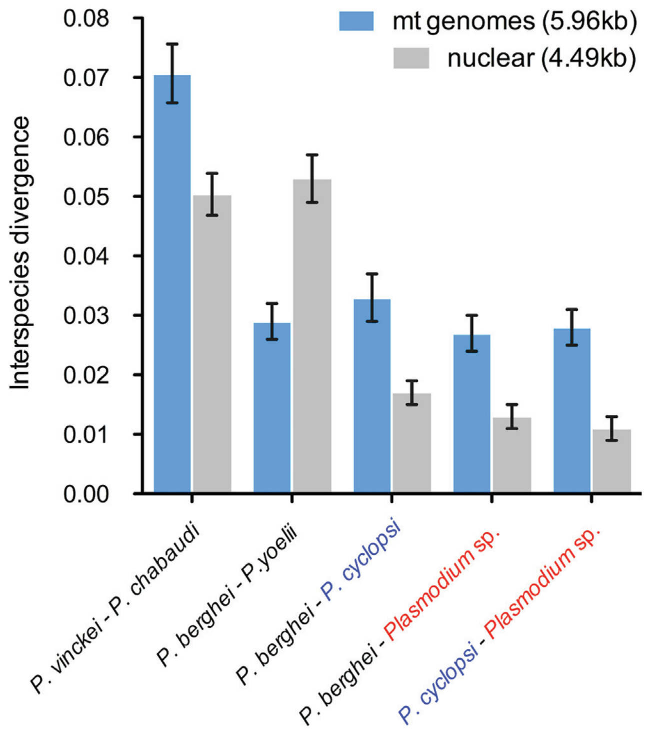

Fig. 3. Overview of estimates of the genetic divergences between selected *Plasmodium* taxa. The number of base substitutions per site between the sequences are shown and standard error estimates are given.

Plasmodium taxa (Supplementary Fig. S6, Supplementary Tables S9–S10). Together, interspecies divergence of mtDNA and selected nuclear genes support a distinct placement of *Plasmodium* sp. (ex *R. landeri*) in the *Vinckeia* clade.

4. Discussion

Bats represent the second largest order within the Class Mammalia, with 1,469 species in 21 families, surpassed in species number only by the order Rodentia (Amador et al., 2018; https://batnames.org/home.html). Among mammals, bats probably host the highest diversity of haemosporidian parasites (Garnham, 1966; Schaer et al., 2013; Perkins and Schaer, 2016); however, only three species of *Plasmodium* have been described, for which scarce reports exist (Table 1).

Traditionally, all bat *Plasmodium* spp. were classified in the subgenus *Vinckeia* with all rodent *Plasmodium* spp. (Garnham, 1966; Landau and Chabaud, 1978). This classification was mainly based on morphological differential characters e.g., the blood schizonts which are relatively small, do not fill the erythrocyte, usually produce eight or fewer merozoites, and lack erythrocyte modifications. *Vinckeia* also includes the two rodent *Plasmodium* sp., *P. atheruri* (host species *Atherurus africanus*, Hystricidae) and *P. anomaluri* (host species *Anomalurus derbianus* (=*fraseri*), Anomaluridae) (van den Berghe et al., 1958; Pringle, 1960), and *Plasmodium* spp. found in Dermoptera (*Plasmodium sandoshami*), Artiodactyla in Africa and Asia (*Plasmodium bubalis, P. brucei, Plasmodium cephalophi, Plasmodium traguli*), and lemurs (Bruce et al., 1913, 1915; Sheather, 1919; Garnham and Edeson, 1962; Dunn et al., 1963; Garnham, 1966). Although there is no molecular data on multiple species, the molecular evidence from ungulates and lemur parasites strongly suggest that *Vinckeia* is polyphyletic (Martinsen et al., 2016; Templeton et al., 2016; Pacheco et al., 2022). Although redescribing or eliminating the subgenus *Vinckeia* is an issue that deserves attention, numerous phylogenetic analyses have found the murine *Plasmodium* spp. as a monophyletic group separated from the two clades of primate-infecting parasites, the subgenera *Plasmodium* (*Plasmodium*) that perhaps also includes lemur parasites (Pacheco et al., 2022) and *Plasmodium* (*Laverania*) that includes *P. falciparum* and parasites from African apes. Further, the two molecular analyses of bat *Plasmodium* spp. confirmed that these parasites share recent common ancestor murine *Plasmodium* spp., *P. berghei and P. yoelii* (Schaer et al., 2013; and this study). In addition, phylogenetic analysis of the *Plasmodium* spp. infecting the African brush-tailed porcupine in the Democratic Republic of the Congo has also confirmed *P. atheruri* as part of this rodent clade (Pacheco et al., 2011). The remaining putative *Vinckeia* members of Rodentia and Dermoptera hosts await molecular verification.

Considering the findings using molecular data, discrepancies with the classical taxonomy could be partially addressed by limiting the use of the subgenus *Vinckeia* to the monophyletic group that includes bats and rodent parasites from Africa. In contrast, other *Plasmodium* spp. such as those found in ungulates and lemurs could be included in different subgenera, assuming that the research community considers the practice of keeping *Plasmodium* subgenera valuable (Pacheco et al., 2022). Thus, we will use the subgenus *Vinckeia* in a very narrow sense, to refer to the *Plasmodium* spp. found in rodents and bats.

In this study, we conducted comprehensive phylogenetic analyses of two *Plasmodium* parasite taxa from bats in the context of rodents and other *Plasmodium* spp. by including complete mtDNA genomes and multiple nuclear markers. The phylogenetic analyses confirmed that *P. cyclopsi* and the rodent parasite *P. berghei* are part of a monophyletic group, with the parasite detected in *R. landeri* as an additional member of the clade that includs *P. cyclopsi* and *P. berghei*, underscoring the shared evolutionary history of these parasites including in the subgenus *Vinckeia*. The exact position of the other bat *Plasmodium* sp., *P. voltaicum,* is not fully resolved yet but it is clearly also part of the rodent/bat *Vinckeia* clade.

The estimates of the genetic divergence between *P. cyclopsi* and *Plasmodium* sp. whole mtDNAs (ex *R. landeri*) (0.028) are compara-

Table 1
Overview of all *Plasmodium* parasite records in bats.

Plasmodium spp.	Bat host species	Prevalence	Country	Reference
P. cyclopsi	***Doryrhina cyclops***	3/5 (60%)	Gabon	Landau and Chabaud, 1978
	Doryrhina cyclops	3/4 (75%)	Liberia	Schaer et al., 2013
	Doryrhina cyclops	**10/10 (100%)**	**Sierra Leone**	**This study**
P. rousetti[a]	*Rousettus aegyptiacus*	7/81 (9%)	Ghana	Riel et al., 1951
P. voltaicum	*Myonycteris angolensis*	25/26 (96%)	Ghana	van der Kaay, 1964
	Myonycteris angolensis	3/3 (100%)	Guinea	Schaer et al., 2013
***Plasmodium* sp.**	***Rhinolophus landeri***	**1/2 (50%)**	**Sierra Leone**	**This study**

Samples used in this study are shown in bold.
[a] No molecular data available.

ble to the estimated number of substitutions between *P. berghei* and *P. yoelii* (0.029), which suggests that the bat *Plasmodium* sp. (ex *R. landeri*) might represent a distinct species. However, the genetic divergence between the nuclear sequences of *P. berghei* and *P. yoelii* were 0.053, while the divergences between *P. cyclopsi*/*P. berghei*, *Plasmodium* sp./*P. berghei* and *P. cyclopsi*/*Plasmodium* sp. were between 0.011 and 0.017, which point to a distinct lineage. Phylogenomic data of the bat *Plasmodium* taxa and additional wild isolates are needed to fully resolve the phylogenetic relationships within the bat/rodent *Plasmodium* group. The *Plasmodium* sp. (ex *R. landeri*) taxon is represented by only a single sample, and subsequent surveys are warranted to characterize this taxon in more detail, including parasite morphology, prevalence, identification of the insect vector, host specificity and geographical distribution.

In this investigation, *P. cyclopsi* represents only the second re-discovery of this species after its first description in Gabon (Landau and Chabaud, 1978) and its first molecular characterization in bats in Liberia (Schaer et al., 2013). In all three cases, *P. cyclopsi* was isolated from *D. cyclops* bat hosts and each time with high prevalences (3/5 individuals infected in Gabon, 3/4 in Liberia, 10/10 in Sierra Leone) (Table 1).

Plasmodium voltaicum of the fruit bat *M. angolensis* is the only other bat *Plasmodium* sp. where molecular data are available (Schaer et al., 2013) and displays the same pattern of narrow bat host specificity with high prevalence (25/26 individuals infected in Ghana, 3/3 in Guinea) (van der Kaay, 1964; Schaer et al., 2013). These data, albeit limited, indicate well-adapted host-parasite systems with efficient transmission cycles (Table 1). It was speculated early on that *Plasmodium* (*Vinckeia*) species in natural environments are usually confined to one vertebrate and one invertebrate host species, which is more host-specific than many of the primate parasites (Garnham, 1966). For instance, the *P. berghei* and *Anopheles dureni* complex is a ‚closed circle' in a highly specialized environment, the mountain range in the eastern Democratic Republic of Congo (Garnham, 1966). Although the invertebrate vectors for all bat *Plasmodium* spp. remain unknown, the vertebrate host specificity for *P. cyclopsi* and *P. voltaicum* can be readily recognized. The third historically described *Plasmodium* sp. from bats, *P. rousetti*, still awaits rediscovery and molecular characterization. The three bat *Plasmodium* spp., *P. voltaicum*, *P. cyclopsi* and *P. rousetti,* have been considered valid species. In contrast, other putative *Plasmodium* spp., reported from bats, were subsequently assigned to other genera (Garnham, 1966). Thus, the discovery of *Plasmodium* parasites in *R. landeri* in this study represents only the fourth *Plasmodium* taxon reported in bats. The finding of *Plasmodium* parasites in a rhinolophid host refutes once more the assumption of *Plasmodium* being strictly limited to fruit bats (Pteropodidae) as initially assumed (Garnham, 1966) and expands the host distribution of *Plasmodium* in bats to four bat species of three closely related bat families (Pteropodidae, Hipposideridae, Rhinolophidae) of the suborder Yinpterochiroptera, indicative of potential co-phylogenetic patterns.

The link between the bat *Plasmodium* spp. might be explained by the striking roosting associations of their respective host species, *D. cyclops*, *M. angolensis* and *R. landeri* (Eisentraut, 1956; Verschuren, 1957, 1967). Their proximity at roosts might expose them to the same dipteran vectors, which could be a decisive ele-

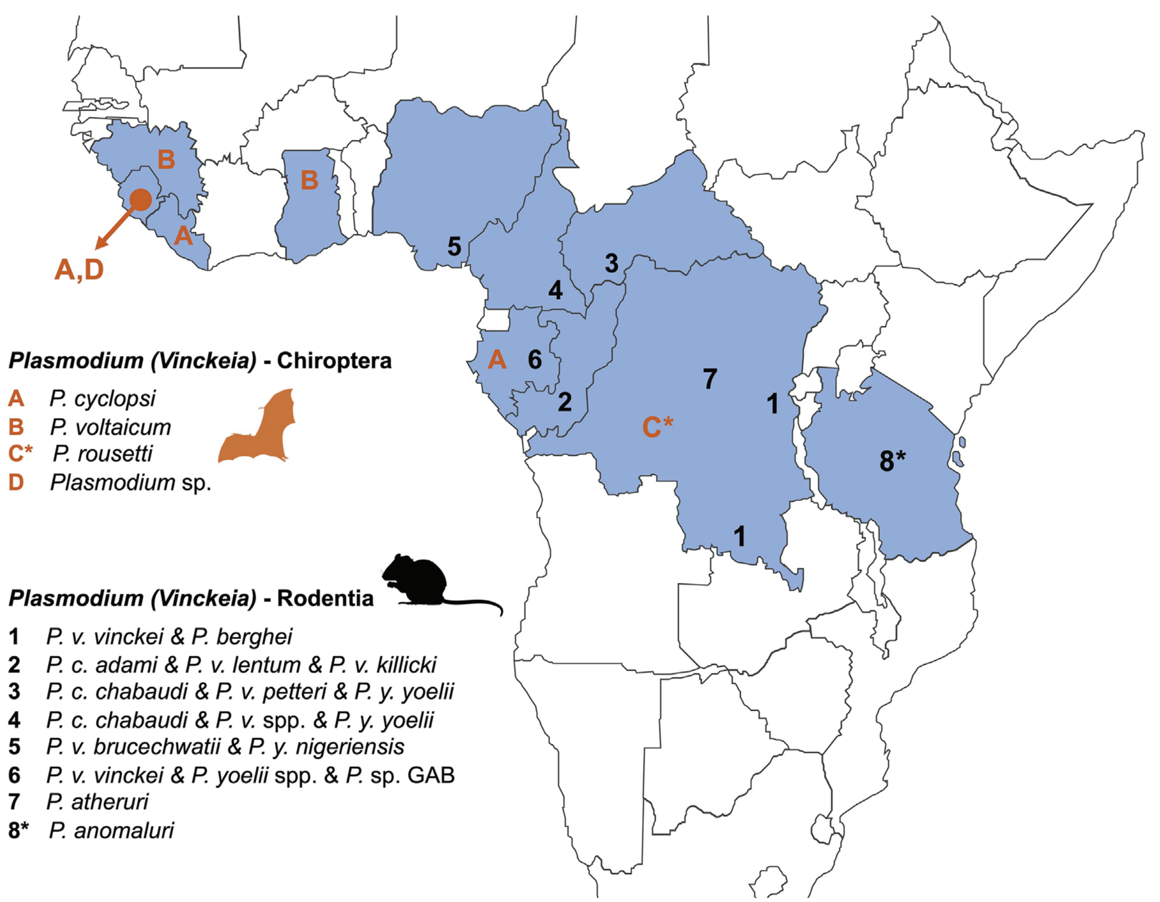

Fig. 4. Distribution of *Plasmodium* (*Vinckeia*) spp. of Chiroptera and Rodentia. Records for rodent *Plasmodium* spp. (Landau and Chabaud, 1994; Boundenga et al., 2019) and bat *Plasmodium* spp. (see Table 1). **Plasmodium* sp. has not yet been verified as species of the subgenus *Vinckeia* by molecular data.

ment in the *Plasmodium* parasites sharing a common ancestor. Similarly, this ecological overlap provides a plausible explanation for the phylogenetic link between rodent and bat *Plasmodium (Vinckeia)* spp. Roosting associations of the bat host species with other mammals include flying squirrels (e.g. *Anomalurus* spp.), and the murid *Praomys tullbergi* (Kuhn, 1962; Rosevear, 1965; Brosset, 1966; Verschuren, 1967; Jones, 1971). Despite limited sampling in small mammals, the relative position of *P. cyclopsi* and *Plasmodium* sp. in the phylogeny is consistent with a host switch from a rodent host to bats.

Another intriguing aspect is the geographic distribution of bat and rodent *Plasmodium* spp. All records of bat *Plasmodium* parasites, including this study, and the *Plasmodium* spp. of the rodent host families Muridae, Hystricidae, and Anomaluridae, have been documented in western and/or central Africa (Garnham, 1966; Landau and Chabaud, 1994; Boundenga et al., 2019) (Table 1, Fig. 4).

This observation raises the possibility that the rodent and bat *Plasmodium* spp. may be limited to this geographical area due to ecological factors, e.g., a narrow distribution of suitable vectors. Indeed, it has been speculated that *Plasmodium* (*Vinckeia*) spp. occupy specialized niches in the tropical forests of the Old World and that even if the mammalian host species features a broader distribution, the infection still seems to be confined to a relatively small area (Garnham, 1966). This pattern may be comparable with the one observed in *Plasmodium* infecting non-human primates in southeastern Asia which seems driven by a combination of vertebrate hosts and suitable vectors (Garnham, 1966; Coatney et al., 1971; Muehlenbein et al., 2015). Only two rodent *Plasmodium* spp., *P. booliati* and *P. watteni* of Asian flying squirrels (*Petaurista* spp., rodent family Sciuridae) that await molecular characterization, are not distributed in the same African area but have been reported from Taiwan and Malaysia (Sandosham et al., 1965; Lien and Cross, 1968). Since *Vinckeia* is not monophyletic (Pacheco et al., 2022) discussions of such predictions seem baseless if the subgenus is considered as initially proposed.

Of all rodent laboratory model systems, *P. berghei* is one of the most widely used. Its closest relatives in bats, *P. voltaicum, P. cyclopsi,* and the potentially new *Plasmodium* sp. of this study, could offer a unique comparative system with the rodent *Plasmodium* spp. Bats and rodents belong to separate evolutionary lineages and, thus, are not closely related (Foley et al., 2023); hence, studying their closely related *Plasmodium* spp. can provide valuable insights into the mechanisms of host switching, host adaptation, and evolutionary dynamics of these parasites.

In conclusion, our findings shed light on the evolutionary relationships of bat *Plasmodium* parasites, their genetic diversity, and distribution. Within this more narrowly defined bat-rodent *Plasmodium* clade, species diversity could be higher than previously reported, as broader taxon sampling in bats might recover additional species. Further investigations are warranted to elucidate the mechanisms driving the diversity, origin, and distribution of these parasites.

CRediT authorship contribution statement

Oskar Werb: Formal analysis, Investigation, Writing – original draft. **Kai Matuschewski:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Validation, Writing – review & editing. **Natalie Weber:** Investigation. **Annika Hillers:** Investigation. **Jerry Garteh:** Investigation. **Amadu Jusu:** Investigation. **Brima S. Turay:** Investigation. **Nadia Wauquier:** Investigation. **Ananias A. Escalante:** Formal analysis, Methodology, Validation, Writing – review & editing. **M. Andreína Pacheco:** Formal analysis, Methodology, Validation, Visualization, Writing – review & editing. **Juliane Schaer:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.ijpara.2024.05.002.

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