First Molecular Detection of *Polychromophilus* Parasites in Brazilian Bat Species

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Abstract: Blood parasites of the Haemosporida order, such as the *Plasmodium* spp. responsible for malaria, have become the focus of many studies in evolutionary biology. However, there is a lack of molecular investigation of haemosporidian parasites of wildlife, such as the genus *Polychromophilus*. Species of this neglected genus exclusively have been described in bats, mainly in Europe, Asia, and Africa, but little is known about its presence and genetic diversity on the American continent. Here, we investigated 406 bats from sites inserted in remnant fragments of the Atlantic Forest and Cerrado biomes and urbanized areas from southern Brazil for the presence of *Polychromophilus* species by PCR of the mitochondrial cytochrome b encoding gene. A total of 1.2% of bats was positive for *Polychromophilus*, providing the first molecular information of these parasites in *Myotis riparius* and *Eptesicus diminutus*, common vespertilionid bats widely distributed in different Brazilian biomes, and *Myotis ruber*, an endangered species. A Bayesian analysis was conducted to reconstruct the phylogenetic relationships between *Polychromophilus* recovered from Brazilian bats and those identified elsewhere. Sequences of Brazilian *Polychromophilus* lineages were placed with *P. murinus* and in a clade distinct from *P. melanipheus*, mainly restricted to bats in the family Vespertilionidae. However, the sequences were split into two minor clades, according to the genus of hosts, indicating that *P. murinus* and a distinct species may be circulating in Brazil. Morphological observations combined with additional molecular studies are needed to conclude and describe these *Polychromophilus* species.

Keywords: *Polychromophilus*; bats; phylogeny; Brazil

1. Introduction

The phylum Apicomplexa forms one of the most diverse groups of unicellular protists with a wide environmental distribution. They are classified as mandatory intracellular parasites and they have mobile invasive stages. They are characterized by the presence of an evolutionarily unique structure called the apical complex, used to adhere and invade host cells. Many of the species that are part of this group are considered pathogens in humans and other vertebrates. All animal species are believed to host at least one species of apicomplexan parasites [1–3]. Apicomplexa are divided into two orders: Eucoccidioida (coccidian parasites) and Haemosporida (haemosporidian parasites). Haemosporida are...
organized into four families: Garniidae, Haemoproteidae, Leucocytozoidae, and Plasmodiidae, which include malaria parasites that infect various vertebrates and invertebrate hosts [4].

The hosts of the order Chiroptera have the greatest diversity of haemosporidian parasites among mammals, including nine genera. In addition to the well-known genera (Plasmodium and Hepatocystis), seven genera exclusively infect chiropterans: Polychromophilus, Nycteria, Bioccala, Biguetiella, Dionisia, Johnsprentia, and Sprattiella [5,6], clearly highlighting this group of mammals as a vital tool in the taxonomic, systematic, and evolutionary study of haemosporidians in mammals. Although Bioccala was elevated to a genus in 1984 [7], many studies, as well as this work, still use it as a subgenus of Polychromophilus, since its species present similar morphological characteristics and its genetics have not been studied [8].

The genus Polychromophilus has been found in insectivorous bats in tropical and temperate regions [9–12]. Only five species of Polychromophilus are known. Although they can be distinguished by slight differences in ultrastructure, they are classified mainly based on the type of host [13]. Of the five species of Polychromophilus described, Polychromophilus (Polychromophilus) melanipherus and Polychromophilus (Bioccala) murinus are mainly linked to two bat families: Miniopteridae and Vespertilionidae, respectively [14]. However, occasionally, P. melanipherus has been reported in Hipposideridae and Vespertilionidae and P. murinus in Rhinolophidae, Hipposideridae, and Miniopteridae [6]. In addition, the species P. (P.) corradetti and P. (P.) adami have been described in bats from the African region: Miniopterus inflatus in Gabon and Miniopterus minor in the Republic of Congo [13].

Recent studies have demonstrated a greater concentration of molecular studies aimed at African and European bats, e.g., [8, 15–17]. In contrast, our knowledge about haemosporidian parasites of Brazilian bats is still restricted to morphological investigations, such as the case of Polychromophilus (Bioccala) deanei found in Myotis nigricans (Vespertilionidae). Myotis nigricans is an evening bat from Brazil, and is the first chiropteran host in which this group of parasites was found in the New World [18,19]. Nevertheless, no molecular data is available for this parasite in Brazil, and the only sequence of Polychromophilus sp. of bats from the American continent is from Myotis nigricans, from the Vespertilionidae family, found in Panama [20].

2. Materials and Methods

2.1. Sampling

Brain tissue samples of bats with no identified species (n = 406) were acquired from the Parana State Reference Laboratory (LACEN) program for monitoring rabies virus circulation. They were collected between September 2019 and August 2020 in 67 different municipalities in the State of Parana, most of them inserted in remnant fragments of Atlantic Forest and Cerrado biomes, as well as in urbanized areas (Figure 1).

All tissue samples and bats were collected and handled under appropriate authorizations by the Brazilian government. The project was approved by the Ethics in Use of Animals Committee, CEUA/SESA, at the Centro de Produção e Pesquisa de Imunobiológicos—CPPI/PR (approval number 01/2019 and date of approval 3 March 2020).
Figure 1. Location of municipalities in the State of Paraná, Brazil, where bat samples were collected.

2.2. Polychromophilus Detection

The extraction of total nucleic acid (DNA and RNA) from collected samples was performed using the BioGene Extraction kit (K204-4, Bioclin, Belo Horizonte, MG, Brazil), following the manufacturer’s instructions.

A fragment of ~1.1 kb (approximately 92% of the gene) from the mitochondrial cytochrome b gene (cytb) was amplified using a nested polymerase chain reaction (PCR), taking standard precautions to prevent cross-contamination of samples. The PCR reactions were conducted as previously described [21] using primers DW2 and DW4 and 5 μL of genomic DNA in the first reaction, and 1 μL aliquot of this product was used as a template for a nested reaction with primers DW1 and DW6.

PCR products were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit in ABI PRISM® 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) using nested PCR primers. The cytb sequences were obtained and aligned with the sequences available at the GenBank® database.

The phylogenetic relationship among reported parasites was inferred using partial cytb gene sequences (1116 bp). GenBank® accessions of the used sequences are shown in the phylogenetic trees. The phylogenetic reconstruction was performed using the Bayesian inference method implemented in MrBayes v3.2.0 [22]. Bayesian inference was executed with two Markov Chain Monte Carlo searches of 3 million generations, with each sampling 1 of 300 trees. After a burn-in of 25%, the remaining 15,002 trees were used to calculate the 50% majority-rule consensus tree. The phylogeny was visualized using FigTree version 1.4.0 [23].

2.3. Host Species Identification

The positive samples were processed using a PCR protocol that amplifies host DNA with primers L14841 and H15149 that were designed to amplify fragments with ~390 bp of the mitochondrial cytb gene from a wide range of animals, including mammals, birds, amphibians, reptiles, and fish [24]. Amplified fragments were sequenced directly using the
corresponding flanking primers. Obtained sequences were compared to other sequences deposited in the GenBank® database (www.ncbi.nlm.nih.gov/blast/Blast.cgi accessed on 19 March 2021). The best close match (BCM) algorithm was used to identify the best barcode matches of a query, and the species name of that barcode was assigned to the query if the barcode was sufficiently similar [25]. Positive identification and host species assignment were made when sequences presented a match of >97%.

Alternatively, for some specimens, a fragment with ~650 bp from the mitochondrial cytochrome c oxidase (coi) gene was amplified by two methods: (i) using the primers VF1_t1 (5′-TGT AAA ACG ACG GCC AGT TCT CAA CCA ACC ACA AAG ACA TTG G-3′) [26] and VR1_t1 (5′-AGG AAA CAG CTA TGA CTA GAC TTC TGG GTG GCC AAA GAA TCA-3′) [27] with PCR conditions and cycling from Kumar et al. [28], and (ii) using the universal primers LCO 1490 and HCO 2198 [29] and PCR protocol based on Ruiz et al. [30].

3. Results

This study detected five samples that were positive for Polychromophilus sp. (sample IDs: 116, 198, 335, 650, and 69642), confirming the presence of parasites of this genus in Brazilian bats. The percentage of positives was 1.2% (5/406) of the number of samples analyzed. Accordingly, the sequences of cytb and coi genes from the positive host samples were from Myotis ruber (116), Myotis riparius (198, 335, and 69642), and Eptesicus diminutus (650), all bats belonging to the Vespertilionidae family, collected in four municipalities in the State of Paraná (Araucária, Cruz Machado, Curitiba, and Pato Branco) (Figure 2). The two samples obtained in Curitiba city were probably from an urban area since Curitiba is the most populous municipality of Paraná state and the eighth in the country.

Figure 2. Distribution of the positive samples of Polychromophilus sp. isolates from Paraná state, Brazil.

The nucleic acid polymorphism in mitochondrial cytb sequences (1116 bp) of Polychromophilus sp. isolates from Brazil compared to the best match sequence from GenBank® (#LN493038 of Myotis nigricans from Panama with 595 bp) is shown in Table 1. Thirteen sites were polymorphic among Brazilian sequences (Table 1). The Panamanian sequence, the only available one obtained from bats from the American continent, showed two nucleic acid substitutions found only in this isolate (gray columns) (Table 1).
Table 1. Nucleic acid polymorphism in mitochondrial cytochrome b gene (cytb) sequences of Polychromophilus sp. isolates from Brazil (116, 198, 335, 650, and 69642) and Panama (MYOPA01).

| Isolate | 219 | 247 | 261 | 273 | 339 | 405 | 512 | 789 | 792 | 810 | 811 | 853 | 885 | 945 | 1086 |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 116     | C   | T   | A   | T   | T   | T   | T   | C   | C   | C   | A   | T   | A   |     |
| 198     | C   | T   | A   | T   | G   | T   | T   | T   | C   | T   | C   | A   | T   |   G |
| 335     | C   | T   | A   | T   | G   | T   | T   | T   | C   | T   | C   | A   | G   |     |
| 650     | T   | T   | C   | A   | A   | C   | T   | C   | T   | C   | T   | T   |   A |
| 69642   | C   | T   | A   | T   | T   | T   | T   | T   | C   | C   | T   | C   | A   |   A |

MYOPA01 has 595 bp and thus, there was no overlap for the nucleotides from 789–1086 with the Brazilian sequences (1116 bp). Gray columns show two nucleic acid substitutions found only in this isolate.

The sequence obtained from bat 650 was the most divergent, with 98–99% of identity with the others (with 11 or 12 nucleic acid substitutions) (Table 2). The Panamanian sequence presented two to eight nucleic acid substitutions compared to Brazilian sequences (98–99% of identity) (Table 2).

Table 2. Similarity percentage between the mitochondrial cytochrome b gene (cytb) sequences of Polychromophilus sp. found in different bats from Brazil and Panama (MYOPA01).

| Bat Species | 116 | 198 | 335 | 650 | 69642 | MYOPA01 |
|-------------|-----|-----|-----|-----|-------|---------|
| 116 Myotis ruber | 1116 | 99% | 99% | 99% | 99% | 99% |
| 198 Myotis riparius | 1113 | 100% | 99% | 99% | 99% | 99% |
| 335 Myotis riparius | 1113 | 1116 | 99% | 99% | 99% | 99% |
| 650 Eptesicus diminutus | 1105 | 1105 | 1105 | 98% | 98% | 98% |
| 69642 Myotis riparius | 1115 | 1112 | 1112 | 1104 | 1116 | 99% |

MYOPA01 Myotis nigricans | 592 | 591 | 591 | 587 | 592 | 595 |

The phylogenetic tree in Figure 3 was generated with reference sequences found in the Genbank® database, covering different haemosporidian genera obtained from different hosts (Table A1, Appendix A). The Polychromophilus sequences found in this study and all sequences of the genus available in the Genbank® database (Table A2, Appendix A) were included. The clade of the genus Polychromophilus is shown in evidence, and the remaining haemosporidian from other genera were collapsed.

Phylogenetic analysis based on cytb did not produce conflict in any of the main nodes. All the main genera and subgenera were recovered and represented in the phylogenetic tree by separate monophyletic clades. The results show the existence of four clades within the Haemosporida order analyzed here. Phylogeny also showed Polychromophilus as a sister clade of a group that contains Plasmodium species of ungulates, but with a distant relationship between Plasmodium and Hepatocystis from other mammals, such as primates and rodents.
Figure 3. Bayesian phylogeny based on the mitochondrial cytochrome b gene (cytb) from Polychromophilus spp. of the sequences identified in the present study (1116 bp) and reference sequences listed in Tables A1 and A2 in Appendix A. * Sequence HM055583 has also been reported in P. murinus from Eptesicus serotinus, Nyctalus noctule, and Myotis myotis (Table A2, Appendix A). Eimeria spp. were used as an external group. The support values of the nodes (in percentage) indicate posterior probabilities. The red branches highlight the haemosporidian sequences found in mammals. The yellow branches highlight the haemosporidian sequences found in birds. The green branches highlight the haemosporidian sequences found in reptiles. The sequences found in the present study are highlighted in bold. The remaining reference sequences are collapsed to highlight the branch of the Polychromophilus genus.

All Polychromophilus sequences from bats of different parts of the world were grouped into a monophyletic clade (posterior probability of 100) composed of four subclades, with all Polychromophilus found in Brazilian bats segregated in only one of them. The first distinct subclade comprised all sequences of P. melanipherus from Miniopterus bat hosts, and the second subclade exclusively included sequences of Polychromophilus from vespertilionids (including Brazilian ones), confirming a clear separation of parasites from miniopterid and vespertilionid hosts. The other subclade that was separated contained the Polychromophilus sequences from Scotophilus kuhlii from Thailand (MT750305-MT750309). Two samples of Pipistrellus aff. grandidieri and Laeophotes capensis from Guinea (KF159700 and KF159714) formed a separate group.

The subclade of Polychromophilus from vespertilionids was divided into two branches: one contained sequences of P. murinus from bats in Europe (Switzerland, Bulgaria), Madagascar, and Thailand, and a sequence of Eptesicus diminutus (650) from Brazil, and the other
clade with *M. nigricans* from Panama and all the other Brazilian sequences isolated from the *Myotis* species.

### 4. Discussion

Based on the results presented herein, although the total number of bat families tested is unknown, *Polychromophilus* infection in Brazilian bats appears to be limited to just one family (Vespertilionidae). This finding is in accordance with the only previous report of *Polychromophilus* from Brazil, described as *P. deanei*, found in *Myotis nigricans*, also a Vespertilionidae bat [18,19].

According to one study, Paraná state has poor fauna regarding the number of bat species, with only 53 species from five families recorded [31]. The Phyllostomidae family has the highest species richness (25; 47% of the total), followed by Molossidae (13; 24%), Vespertilionidae (12; 22%), Noctilionidae (2; 4%), and Emballonuridae (1; 2.5%) [31]. Miretzki also showed the occurrence of only 55% of the species of the Atlantic Forest biome and the relative predominance of vespertilionids and molossids over phyllostomids. Herein, we analyzed samples obtained from much of the state’s area, with great sampling opportunities for other families. However, we were unable to find *Polychromophilus* in bat species that were not vespertilionids, suggesting that this parasite may be restricted to this group of bats in Brazil.

Regarding the frequency, we found the lowest positivity rate reported to date, although the total number of samples analyzed herein is one of the highest among published studies (Table 3). This could be related to the sample type analyzed in this study. This was the first time that *Polychromophilus* DNA was obtained from brain tissue, probably from parasites in the blood vessels that irrigate the organ. Thus, the direct comparison of the prevalence data with published studies that used blood samples is impaired.

### Table 3. Occurrence of *Polychromophilus* sp. in this study and previous studies worldwide.

| Country or Continent | Analyzed Samples | Positive Samples (Positivity) | Positive Host Species | Reference |
|----------------------|------------------|-------------------------------|-----------------------|-----------|
| Africa 1             | 505              | 56 (11%)                      | *Miniopterus africana*, *M. fraterculus*, *M. minor*, *M. natalensis*, *M. rufus*, *Myotis tricolor* | [17]      |
| Australia 2           | 85               | 47 (55%)                      | *Eptesicus diminutus*, *Myotis ruber*, *Myotis riparius* | [32]      |
| Brazil 3              | 406              | 5 (1.2%)                      |                       | This study |
| Europe 4              | 310              | 231 (74.5%)                   | *Miniopterus schreibersii* | [33]      |
| Gabon                 | 164              | 5 (3%)                        | *Miniopterus inflatus* | [34]      |
| Gabon                 | 92               | 2 (2%)                        | *Miniopterus minor*    | [35]      |
| Guinea                | 274              | 5 (2%)                        | *Miniopterus villiersi*, *Neoromicia capensis*, *Pipistrellus aff. grandilaberi* | [15]      |
| Madagascar            | 947              | 130 (13.5%)                   | *Paratriaenops furculus*, *Miniopterus aellenti*, *M. manavi*, *M. gleni*, *M. griffithi*, *M. griveaudi*, *M. mahafaliensis*, *M. majori*, *M. sororculus*, *Myotis goudoti* | [36]      |
Three different Brazilian bats species were found to be positive for *Polychromophilus* sp.: two *Myotis* species (*M. ruber* and *M. riparius*) and one species from the *Eptesicus* genus (*E. diminutus*). There are reports of *Myotis* species infections in Africa (*M. tricolor* in Kenya and *M. goudoti* in Madagascar) [17,36,37], Europe (*M. daubentonii* and *M. myotis* in Switzerland) [38], and Asia (*M. siligorensis* in Thailand) [40]. However, the only record of *Polychromophilus* infection in *Eptesicus* comes from Europe (*E. serotinus* in Switzerland) [38].

*Myotis riparius* is present in Honduras, Uruguay, Bolivia, Argentina, Paraguay, Trinidad, and Brazil [41], including the state of Paraná [31,42,43]. *Myotis ruber* is an endangered species under the category of “vulnerable” according to the Brazilian Institute of Environment and Renewable Natural Resources—IBAMA [44], and under the category of “near threatened” at a global level according to IUCN [45]. It is distributed across Argentina, Uruguay, Paraguay [40,46–48], and southeastern Brazil, including Paraná [49].

It is important to note that in our molecular identification of the host species using *cytb* and sequence comparisons, *Eptesicus furinalis* was the species with the best close match with the sequence obtained from bat 650. However, the percentage of identity was low (89%) compared to sequences available in the GenBank® database, making it impossible to identify the species. Thus, alternatively, we used the *coi* gene and the BOLD database (https://www.boldsystems.org/ (accessed on 31 March 2021), finding 98% of identity with an *Eptesicus diminutus* sequence, a reliable value for the species identification using the BCM method.

Our phylogenetic analysis showed a strongly defined clade represented by *Plasmodium* infecting rodents and primate hosts, which also included *Hepatocystis* isolated from bats. Similar data were obtained by other authors [38,50]. *Haemoproteus* and *Leucocytozoon* species were grouped separately in individual clades, as previously shown [51,52].

Regarding *Polychromophilus* sequences, a similar topology in the phylogenetic tree was obtained by Chumnandee et al. [39], where they grouped into a monophyletic clade with a clear separation of parasites from minipterid and vespertilionid hosts. Four Brazilian sequences (GenBank® MW984519, MW984520, MW984522 from *Polychromophilus* sp. isolated of *Myotis riparius*, and MW984518 from *Polychromophilus* sp. isolated of *Myotis ruber*) were positioned close to the sequence of *Polychromophilus* sp. of bats of the species *Myotis nigricans*, Vespertilionidae family, from the Latin American region (Panama) (GenBank® #LN483038) [20]. One Brazilian sequence (GenBank® #MW984521, from *Polychromophilus* isolated from *Eptesicus diminutus*) was grouped with all *P. murinus*
sequences in a sister clade. The latter, likely *P. murinus*, presented 1% divergence in the *cytb* sequence compared to the other Brazilian or Panamanian sequences, and was obtained from a different genus of bats. Thus, the possibility of most Brazilian sequences being a different *Polychromophilus* species must be investigated.

The present study provides the first molecular description of *Polychromophilus* parasites in *Myotis ruber*, *Myotis riparius*, and *Eptesicus diminutus* from Brazil and confirms the presence of this parasite 50 years after its first and only report in Brazilian territory. Moreover, our results suggest the occurrence of two distinct *Polychromophilus* species infecting two different genera of hosts, improving the current knowledge on blood parasites infecting Brazilian bats. However, it is crucial to add additional molecular markers to the phylogenetic analysis for an in-depth investigation. A three-genome phylogenetic analysis for robust haemosporidian phylogenies has been recommended [53] and must be properly included as part of a follow-up paper. Moreover, additional studies including morphological observations of these parasites combined with molecular data are needed to resolve its taxonomy. Furthermore, due to the great Brazilian extensions and the immense diversity of species and biomes, new bat populations should be investigated to provide a complete portrait of the biology of host–parasite interactions.

**Author Contributions:** Conceptualization, A.W.B. and K.K.; formal analysis, B.d.S.M. and C.C.d.A.; investigation, B.d.S.M., L.d.O.G., C.C.d.A. and E.F.M.; resources, G.A.M., I.N.R. and K.K.; data curation, B.d.S.M. and C.C.d.A.; writing—original draft preparation, B.d.S.M., A.P.d.S. and K.K.; writing—review and editing, G.A.M., B.d.S.M., I.N.R., L.d.O.G., C.C.d.A., E.F.M., A.P.d.S., A.W.B. and K.K.; visualization, B.d.S.M. and C.C.d.A.; supervision, K.K.; project administration, K.K.; funding acquisition, K.K. All authors have read and agreed to the published version of the manuscript.

**Funding:** B.d.S.M. and C.C.d.A. are currently funded by a master scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—CAPES (process numbers 88887.463661/2019-00 and 88887.463659./2019-00). K.K. is a CNPq research fellow (process number 308678/2018-4). L.d.O.G. is supported by a postdoctoral fellowship (FAPESP 2018/16232-1). This research benefited from the State Research Institutes Modernization Program, funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2017/50345-5).

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics in Use of Animals Committee, CEUA/SESA, of the Centro de Produção e Pesquisa de Imunobiológicos—CPPI/PR (approval number 01/2019 and date of approval 3 March 2020).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available in Appendix A and also in the GenBank® database (https://www.ncbi.nlm.nih.gov/genbank/ (accessed on 19 March 2021)) (accession numbers MW984518-MW984522).

**Conflicts of Interest:** The authors declare no conflict of interest.
Appendix A

Table A1. Mitochondrial cytochrome b gene (cytb) sequences of the parasite used as references for phylogenetic analyses and their respective accession numbers in the Genbank® database.

| GenBank® Accession Number | Parasite Species         | Host          |
|---------------------------|--------------------------|---------------|
| HQ173882                  | *Eimeria magna*          | Rabbit        |
| HQ173892                  | *Eimeria vejdovskyi*     | Rabbit        |
| AY099045                  | *Haemoproteus majoris*   | Bird          |
| HM222472                  | *Haemoproteus sp.*       | Bird          |
| KT367832, KT367833, KT367822, KT367828 | *Haemosporida sp.*       | Antelope      |
| KT367830, KT367819, KT367837 | *Haemosporida sp.*       | Antelope      |
| FJ168565                  | *Hepatocystis sp.*      | Bat           |
| JQ070951, JQ070956        | *Hepatocystis sp.*      | Monkey        |
| AY099063                  | *Leucocytozoon dubreuli*| Bird          |
| NC_012450, FJ168563       | *Leucocytozoon majoris* | Bird          |
| KF159690, KF159720, MK098843-MK098847 | *Nycteris sp.*     | Bat           |
| GQ141581, GQ141585, KT367845, KM598212 | *Parahaemoproteus sp.* | Bird          |
| NC_012447, FJ168561       | *Parahaemoproteus vireonis* | Bird          |
| HM235081                  | *Plasmodium adleri*      | Gorilla       |
| AY099054, HQ712051        | *Plasmodium atherari*    | Rodent        |
| AY099055                  | *Plasmodium azurophilum*| Lizard        |
| KP875474                  | *Plasmodium billcollinsi*| Chimpanzee    |
| HM235065                  | *Plasmodium blacklocki* | Gorilla       |
| KF159674                  | *Plasmodium cyclopsi*    | Bat           |
| AB444126                  | *Plasmodium cynomolgi*   | Monkey        |
| FJ895307                  | *Plasmodium gaboni*      | Chimpanzee    |
| AF069612                  | *Plasmodium gallinaceum* | Bird          |
| AY099053                  | *Plasmodium giganteum*   | Lizard        |
| JF923751                  | *Plasmodium gonderi*     | Mandrill      |
| JQ345504                  | *Plasmodium knowlesi*    | Human         |
| HM000110                  | *Plasmodium malariae*    | Chimpanzee    |
| GU723548                  | *Plasmodium ovale*       | Human         |
| JF923762                  | *Plasmodium praefalciparum* | Monkey      |
| KP875479                  | *Plasmodium reichenowi*  | Chimpanzee    |
| AY733090                  | *Plasmodium relictum*    | Bird          |
| HM222485                  | *Plasmodium sp.*         | Bird          |
| JF923753                  | *Plasmodium sp.*         | Mandrill      |
| KJ700853, KJ700854        | *Plasmodium vincke*      | Rodent        |
| KF591834                  | *Plasmodium vivax*       | Human         |
| KF159671                  | *Plasmodium volcaeformis*| Bat           |
| DQ414658                  | *Plasmodium yoelii killicki* | Rodent    |
Table A2. Genbank® accession numbers of Polychromophilus mitochondrial cytochrome b gene (cytb) sequences used as a reference for phylogenetic analyses and sequences found in this study.

| GenBank Accession Number | Parasite Species | Host | Origin |
|--------------------------|------------------|------|--------|
| KU318045                 | *P. melanipherus*| *Anopheles marshalli* | Gabon |
| HM055583                 | *P. murinus*     | *Myotis daubentonii*   | Switzerland |
| HM055583                 | *P. murinus*     | *Eptesicus serotinus*   | Switzerland |
| HM055583                 | *P. murinus*     | *Nyctalus noctula*     | Switzerland |
| HM055583                 | *P. murinus*     | *Myotis myotis*        | Switzerland |
| HM055584-HM055589        | *P. murinus*     | *Myotis daubentonii*   | Switzerland |
| MW984521                 | *Polychromophilus* sp. | *Eptesicus diminutus*   | Brazil (this study) |
| KT750375                 | *Polychromophilus* sp. | *Miniopterus africanus* | Kenya |
| MH744509–MH744511, MH744518, MH744521 | *P. melanipherus* | *Miniopterus griveaudi* | Madagascar |
| MH744506, MH744519       | *P. melanipherus* | *Miniopterus griveaudi* | Madagascar |
| MH744514–MH744516        | *P. melanipherus* | *Miniopterus griveaudi* | Madagascar |
| MH744508, MH744522–MH744525 | *P. melanipherus* | *Miniopterus griveaudi* | Madagascar |
| JQ995284–JQ995288       | *Polychromophilus* sp. | *Miniopterus inflatus* | Gabon |
| MH744504, MH744505       | *P. melanipherus* | *Miniopterus mahafalensis* | Madagascar |
| MH744512, MH744526       | *P. melanipherus* | *Miniopterus manavi* | Madagascar |
| KT750430                 | *Polychromophilus* sp. | *Miniopterus minor* | Tanzania |
| MK098848, MK098849       | *Polychromophilus* sp. | *Miniopterus minor* | Gabon |
| MW007677                 | *P. melanipherus* | *Miniopterus natalensis* | South Africa |
| KT750376-KT750382, KT750401, KT750402 | *Polychromophilus* sp. | *Miniopterus natalensis* | Kenya |
| KT750406, KT750408, KT750409 | *Polychromophilus* sp. | *Miniopterus natalensis* | Kenya |
| MK088162–MK088168       | *P. melanipherus* | *Miniopterus orianae* | Australia |
| KT750383-KT750386, KT750415, KT750418 | *Polychromophilus* sp. | *Miniopterus rufus* | Kenya |
| JN990708–JN990711       | *P. melanipherus* | *Miniopterus schreibersii* | Switzerland |
| KJ131270–KJ131277       | *P. melanipherus* | *Miniopterus schreibersii* | Southern and Central Europe |
| MW007689                 | *P. melanipherus* | *Miniopterus schreibersii* | Spain |
| KT750389                 | *Polychromophilus* sp. | *Miniopterus sp.* | Tanzania |
| KT750387                 | *Polychromophilus* sp. | *Miniopterus sp.* | Kenya |
| KF159675, KF159681, KF159699 | *Polychromophilus* sp. | *Miniopterus villiersi* | Guinea |
| JN990712, JN990713       | *P. murinus*     | *Myotis daubentonii* | Switzerland |
| MH744532–MH744536       | *P. murinus*     | *Myotis goudoti*       | Madagascar |
| LN483038                 | *Polychromophilus* sp. | *Myotis nigricans* | Panamá |
| MW984519, MW984520, MW984522 | *Polychromophilus* sp. | *Myotis riparius* | Brazil (this study) |
| MW984518                 | *Polychromophilus* sp. | *Myotis ruber* | Brazil (this study) |
| GenBank Accession Number | Parasite Species       | Host                  | Origin   |
|--------------------------|------------------------|-----------------------|----------|
| MT136168                 | *P. murinus*           | *Myotis siligorensis* | Thailand |
| KF159700                 | *Polychromophilus* sp. | *Neoromicia capensis* | Guinea   |
| MW007685                 | *P. melanipherus*      | *Nycteribia schmidlii*| Spain    |
| MW007680, MW007681       | *P. melanipherus*      | *Nycteribia schmidlii*| Hungary  |
| MW007682                 | *P. melanipherus*      | *Nycteribia schmidlii*| Italy    |
| MW007671–MW007674, MW007676 | *P. melanipherus* | *Nycteribia schmidlii scotti* | South Africa |
| KU182361–KU182367       | *P. murinus*           | *Rhinolophus* sp.     | Bulgaria |
| KU182368                 | *P. murinus*           | *Penicillidia fulvida*| Gabon    |
| MH744528–MH744531       | *P. melanipherus*      | *Penicillidia leptothrinx* | Madagascar |
| MH744537                 | *P. murinus*           | *Penicillidia sp.*    | Madagascar |
| LN483036                 | *P. murinus*           | *Rhinolophus* sp.     | Bulgaria |
| MT750305–MT750309       | *Polychromophilus* sp. | *Scotophilus kuhlii*  | Thailand |
| MT136167                 | *P. melanipherus*      | *Taphozous melanopogon* | Thailand |

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