

# Phylogeny of *Hepatocystis* parasites of Australian flying foxes reveals distinct parasite clade<sup>☆</sup>

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## ABSTRACT

*Hepatocystis* parasites are close relatives of mammalian *Plasmodium* species and infect a range of primates and bats. Here, we present the phylogenetic relationships of *Hepatocystis* parasites of three Australian flying fox species. Multilocus phylogenetic analysis revealed that *Hepatocystis* parasites of *Pteropus* species from Australia and Asia form a distinct clade that is sister to all other *Hepatocystis* parasites of primates and bats from Africa and Asia. No patterns of host specificity were recovered within the *Pteropus*-specific parasite clade and the *Hepatocystis* sequences from all three Australian host species sampled fell into two divergent clades.

## 1. Introduction

The life-threatening disease malaria is caused by single-cell eukaryotic parasites of the genus *Plasmodium*. The human-infecting *Plasmodium* species belong to a large monophyletic group of haemosporidian parasites (Haemosporida) that comprise 200 formally described species of *Plasmodium* and almost 300 closely related species (Martinsen and Perkins, 2013; Galen et al., 2018). These parasites infect a diverse array of dipteran invertebrate as well as vertebrate hosts. Mammalian haemosporidian parasites are currently classified in ten different genera, including *Plasmodium* and *Hepatocystis*, whereas the mammalian *Plasmodium* clade is paraphyletic, because it contains the parasites of the genus *Hepatocystis* (Perkins and Schaer, 2016; Galen et al., 2018). The large group of haemosporidian parasites not only differs in host preferences, but also in morphology, life history and effects on their hosts. Uncovering the evolutionary history and biology of these parasites will help define their biological adaptations, host switches and acquisitions of novel life-history traits. For example, erythrocytic schizogony or the multiplication of parasites in the blood, the life cycle stage that is associated with the characteristic clinical signs of malaria in humans, is exclusive to parasites of the genus *Plasmodium* (that infect several groups of vertebrates) and is absent in all

other haemosporidian parasite genera.

Bats (Chiroptera) are hosts to perhaps the most diverse array of haemosporidian parasites among mammals, with nine different genera described in this mammalian order. The parasite genera in bats comprise species of *Plasmodium* and *Hepatocystis*, and seven genera thought to exclusively infect chiropteran hosts (Perkins and Schaer, 2016). Due to this diversity, bat malaria parasites are of particular interest for the study of the taxonomy and systematics of malaria parasites. Recent molecular studies have mainly focused on bat malaria parasites from African and European bat hosts (e.g. Witsenburg et al., 2012; Schaer et al., 2013; Lutz et al., 2016; Schaer et al., 2017). In contrast, our knowledge of haemosporidian parasites of Australian bats is very limited and restricted to morphological investigations, with no molecular data available for any of the haemosporidian parasites in these bats. Molecular studies have greatly improved our understanding of haemosporidian parasites. They are particularly needed for the characterization of the understudied haemosporidians of wildlife to recover the evolutionary history of key life-history characters of the whole group, to determine host specificity patterns and the transitions among the different dipteran and vertebrate host groups (Martinsen and Perkins, 2013). Further, molecular identifications allow the differentiation of parasites that are morphologically indistinguishable and are essential

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for any taxonomic revisions of described morphospecies (Galen et al., 2018). Based on morphological criteria, four haemosporidian genera have been reported in Australian bat hosts: *Hepaticystis*, *Polychromophilus*, *Johnspretia* and *Sprattiella* (Table S1). The conception of the two genera *Johnspretia* and *Sprattiella* has only been introduced very recently (Landau et al., 2012a, b). The parasite observations that led to the description of these two new parasite genera were gathered from infected *Pteropus alecto* individuals collected in Townsville, Queensland, Australia in 1978. Both *Johnspretia* and *Sprattiella* await independent confirmation. *Polychromophilus* parasites only infect insectivorous bats of the bat families Miniopteridae and Vespertilionidae (suborder Yangochiroptera) and display a wide distribution in temperate and tropical areas worldwide. In contrast, *Hepaticystis* is restricted to two bat families of the suborder Yinpterochiroptera, fruit bat/flying fox species (Pteropodidae) and hipposiderid bats (Hipposideridae) of Africa, Asia and Australia (c.f. Perkins and Schaer, 2016). The first reports of *Hepaticystis* in Australian flying foxes date back to 1909 and 1911, with “*Plasmodium*” infections independently reported in *Pteropus alecto gouldi* (Breinl et al., 1912). The parasite species was originally described as *Plasmodium pteropi*, and later reclassified as *Hepaticystis pteropi* (Manwell, 1946; Garnham, 1966). Four Australian flying fox species of the genus *Pteropus* (*Pteropus alecto*, *Pteropus poliocephalus*, *Pteropus scapulatus*, *Pteropus conspicillatus*) were subsequently identified as hosts for *H. pteropi* (Mackerras, 1959). A second species of *Hepaticystis*, *Hepaticystis levinei* was described from *P. poliocephalus* (Landau et al., 1985) and more recently, a study reported the same species from *P. alecto*, sampled in Queensland in 1978 (Landau et al., 2012a). Mammalian haemosporidian genera that lack asexual replication in the blood (all haemosporidian genera except *Plasmodium*) are generally considered as benign infections in their mammalian hosts. The blood stages of *Hepaticystis*, *Sprattiella* and *Johnspretia* parasites are confined to gametocytes, whereas the schizonts occur in different tissues. According to the authors, *Sprattiella*-infected bats feature schizonts in the lumen of renal veins of the kidney and *Johnspretia* parasites develop schizonts in the lungs (Landau et al., 2012a,b), whereas schizont stages of *Hepaticystis* are limited to hepatocytes (Landau et al., 2012a,b).

This study investigated the phylogenetic relationships of haemosporidian parasites of three mainland Australian flying fox species.

## 2. Material and methods

### 2.1. Sampling

Samples collected by Edson et al. (2015) and McMichael et al. (2017) were provided by Biosecurity Queensland, Department of Agriculture, Fisheries and Forestry under agreement. Molecular data of haemosporidian parasites was generated from a total of forty infected individuals, which included the three bat host species, *P. alecto* (“Black flying fox”;  $n = 11$ ), *P. conspicillatus* (“Spectacled flying fox”;  $n = 21$ ) and *P. scapulatus* (“Little red flying fox”;  $n = 8$ ) (Table 1).

### 2.2. Microscopy

The blood stages of haemosporidian parasites were investigated and compared to formally described haemosporidian morphospecies using light microscopy with oil immersion and a magnification at 1,000 $\times$ . Where thin blood smears were available, they were fixed in 100% methanol and stained with Giemsa.

### 2.3. Molecular methods and phylogenetic analyses

DNA was extracted from dried blood dots on FTA cards (GE Healthcare) or from organ tissues (lung, liver) using the QIAGEN DNeasy blood and tissue extraction kit (Hilden, Germany). Extractions followed the protocol for animal tissues, and samples were eluted in 60–100  $\mu$ l AE buffer (depending on the intensity of the blood dots).

**Table 1**

List of investigated samples and corresponding blood stage morphology.

<sup>a</sup> Sample/Abbreviation in phylogenetic trees	Locality	<sup>b</sup> blood stage morphology
<i>P. alecto</i> _A17	Ayr	No blood smear available
<i>P. alecto</i> _A22	Tallebudgera	No blood smear available
<i>P. alecto</i> _L1	Boonah	Category 2
<i>P. alecto</i> _L2	Boonah	Subpatent infection, no parasites detected
<i>P. alecto</i> _L3	Boonah	Category 2
<i>P. alecto</i> _L5	Boonah	Category 2
<i>P. alecto</i> _L7	Boonah	No blood smear available
<i>P. alecto</i> _L8	Boonah	Category 2
<i>P. alecto</i> _L10	Boonah	No blood smear available
<i>P. alecto</i> _L11	Boonah	No blood smear available
<i>P. alecto</i> _L13	Boonah	No blood smear available
<i>P. conspicillatus</i> _A25	Topaz	No blood smear available
<i>P. conspicillatus</i> _L14	Gordonvale	Category 1
<i>P. conspicillatus</i> _L15	Gordonvale	Category 1
<i>P. conspicillatus</i> _L16	Gordonvale	Category 1
<i>P. conspicillatus</i> _L17	Gordonvale	Category 1
<i>P. conspicillatus</i> _L18	Gordonvale	Category 1
<i>P. conspicillatus</i> _L19	Gordonvale	Category 1
<i>P. conspicillatus</i> _L20	Gordonvale	Category 1
<i>P. conspicillatus</i> _L21	Gordonvale	Category 1
<i>P. conspicillatus</i> _L22	Gordonvale	No blood smear available
<i>P. conspicillatus</i> _L23	Gordonvale	No blood smear available
<i>P. conspicillatus</i> _L25	Gordonvale	No blood smear available
<i>P. conspicillatus</i> _L27	Gordonvale	No blood smear available
<i>P. conspicillatus</i> _L32	Gordonvale	No blood smear available
<i>P. conspicillatus</i> _L33	Gordonvale	No blood smear available
<i>P. conspicillatus</i> _L34	Gordonvale	No blood smear available
<i>P. conspicillatus</i> _L35	Gordonvale	No blood smear available
<i>P. conspicillatus</i> _L37	Gordonvale	Category 2
<i>P. conspicillatus</i> _L40	Gordonvale	Category 2
<i>P. conspicillatus</i> _L41	Gordonvale	No blood smear available
<i>P. conspicillatus</i> _L43	Gordonvale	Category 1
<i>P. scapulatus</i> _A1	North Lakes	No blood smear available
<i>P. scapulatus</i> _A2	Caboolture	No blood smear available
<i>P. scapulatus</i> _A3	Gatton	Category 1 (plus possible mixed infection with <i>Johnspretia</i> )
<i>P. scapulatus</i> _A4	Mitchell	Category 1 (plus possible mixed infection with <i>Johnspretia</i> )
<i>P. scapulatus</i> _A6	Whiteside	Subpatent infection, no parasites detected
<i>P. scapulatus</i> _A7	Middle Park	No blood smear available
<i>P. scapulatus</i> _A20	Mooloolaba	Subpatent infection, no parasites detected
<i>P. scapulatus</i> _A23	Queensland	Subpatent infection, no parasites detected

<sup>a</sup> All samples were positive by PCR analysis.

<sup>b</sup> Morphology was assigned to categories 1 (*Hepaticystis levinei/pteropi*) and 2 (*Hepaticystis* sp., Landau et al., 2012a), as described in the main text.

Five genes from the three parasite genomes were targeted for subsequent phylogenetic analysis: the two mitochondrial genes cytochrome *b* (*cytb*) and cytochrome oxidase 1 (*cox1*); one apicoplast gene, caseinolytic protease gene (*clpC*); and the two nuclear genes elongation factor 2 (*EF2*) and pantothenate transporter (*PAT*). PCR primers and corresponding cycling protocols followed Martinsen et al. (2008); nested PCR approaches for *cox1* and *clpC*, Schaer et al. (2013); *cytb* and *EF2*, Perkins and Schall (2002); *cytb* and Borner et al. (2016) and Galen et al. (2018); nested PCR approach for *PAT*). All PCR products were sequenced in both directions using BigDye v3.0 (Applied Biosystems) and run on an ABI-373 sequencer (primers and GenBank accession numbers listed in Tables S2 and S3). PCR primers were used for sequencing with the exception of *PAT*, where the inner primers for the nested PCR incorporated CAG or M13R tags, which were used for subsequent sequencing, following Galen et al. (2018). The gene sequences revealed some genetically mixed infections (different haplotypes), visible as double nucleotide peaks in the sequence electropherograms, which has

also been reported in *Hepatocystis* infections in African bats and in primates (Thurber et al., 2013; Schaer et al., 2017). We amplified each locus one to three times per parasite sample and sequenced from both directions. If double peaks could not be resolved in the individual sequence assemblies and still contained more than about three double peaks across the partial gene sequence, we scored the infection as a mixed haplotype infection and excluded this sample from the subsequent phylogenetic analysis. Ambiguous base calls due to sequencing errors in sequences of lower quality were coded as any base (N).

Sequence data were combined with published sequences for the haemosporidian taxa *Leucocytozoon*, *Haemoproteus*, *Plasmodium*, *Nycteria*, *Polychromophilus* and *Hepatocystis*, comprising representatives of all mammal-infecting haemosporidian taxa (where sequences are available) as well as the more distantly related bird-infecting major haemosporidian clades (Table S3). Sequences were assembled and aligned in Geneious 8.1.9 using MUSCLE (Edgar, 2004) with the exception of the PAT sequences, which were aligned using MAFFT version 7 (Kuraku et al., 2013; Katoh et al., 2017) following Galen et al. (2018). The gene alignments were concatenated in Geneious. The concatenated alignment comprised a total length of 3683bp (including 906bp of cytochrome *b*, 951bp of cytochrome oxidase I, 528bp of the apicoplast *clpC*, 516bp of the nuclear *EF2* gene and 782bp of the nuclear PAT gene). Concatenation of the individual gene sequence alignments into a multi-gene alignment for subsequent phylogenetic analysis were chosen over species tree methods due to the high amount of missing data of the published reference sequences at the apicoplast and nuclear loci. Concatenation has been shown to be more robust to the effects of missing sequence data (e.g. Hovmöller et al., 2013; Jiang et al., 2014). The best partitioning schemes and models for the phylogenetic analysis of the concatenated alignment were evaluated with the software *PartitionFinder v.2* (Lanfear et al., 2017) via the CIPRES Science Gateway Web Portal V3.3 (Miller et al., 2010) (Schaer et al., 2017; Tables S4 and S5). Phylogenetic relationships were evaluated by using Bayesian inference and maximum-likelihood (ML) methods and *Leucocytozoon* as outgroup taxon (Borner et al., 2016). *RaxmlGUI* (Silvestro and Michalak, 2012) was used for the ML analysis and nodal support was evaluated using 100 thorough bootstrap pseudoreplicates (Stamatakis et al., 2008). *MrBayes v3.2.6* (Huelsenbeck and Ronquist, 2001) via the CIPRES Science Gateway Web Portal V3.3 (Miller et al., 2010) was used for Bayesian inference, with two runs of four chains (three heated, one cold, temperature = 0.02) each for 25 million generations. The first 25% of trees were discarded as burn-in. Mixing and convergence of runs, and effective sample size (ESS > 1000) were valued with Tracer v1.6 (Rambaut et al., 2014) and phylogenetic trees were displayed in *FigTree* (<http://tree.bio.ed.ac.uk/software/figtree/>).

### 3. Results

#### 3.1. Phylogenetic analysis reveals distinct clade of *Hepatocystis*

The maximum likelihood and Bayesian analysis of the concatenated dataset of five genes, rooted with the avian haemosporidian genus *Leucocytozoon*, confirmed *Hepatocystis* as monophyletic clade with high support and grouped all sequences isolated from the parasites of the three different Australian *Pteropus* host species within the *Hepatocystis* clade (Fig. 1). The Bayesian analysis recovered the mammalian *Plasmodium* (*Plasmodium*) and *Plasmodium* (*Vinckeia*) clade as closest relative of *Hepatocystis*, as shown before (e.g. Borner et al., 2016; Galen et al., 2018). Strikingly, within the *Hepatocystis* clade, the Australian and Asian parasite sequences from *Pteropus* hosts form one distinct clade, which groups as sister clade to the remaining primate and bat *Hepatocystis* sequences (Fig. 1). Moreover, the “*Pteropus*” parasite clade is separated in two groups, each comprising a mix of parasite sequences of the different *Pteropus* host species. One group includes the published parasite sequences of the Asian bat hosts *Pteropus vampyrus* and *Pteropus hypomelanus*, and the other also contains one parasite sequence of the

Asian *P. vampyrus* host (Fig. 1). These data clearly suspend host-specificity patterns.

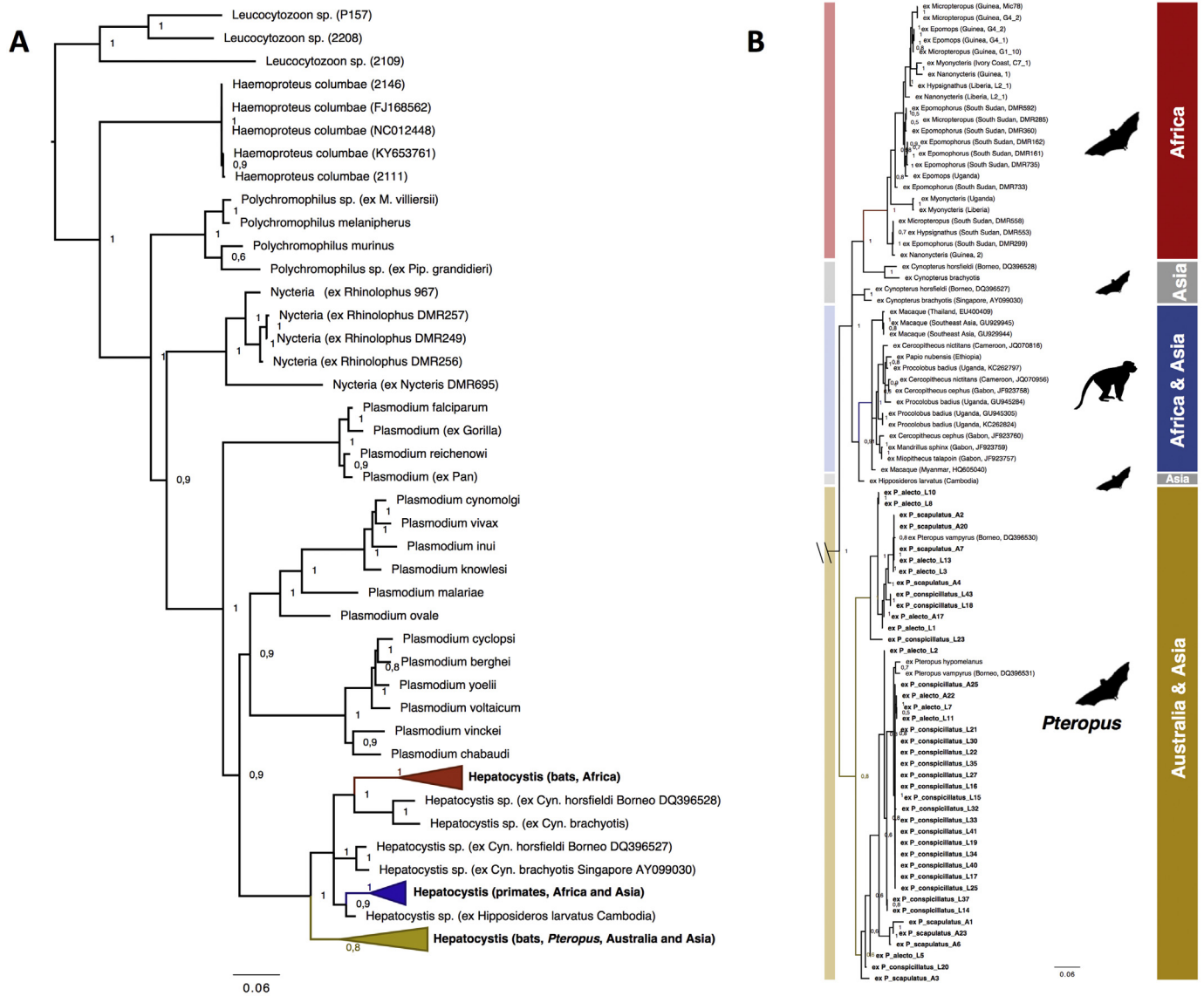
Together, the chiropteran/primate *Hepatocystis* sister clade contains the monophyletic primate *Hepatocystis* clade with the single parasite sequence of the Asian bat *Hipposideros larvatus* and two parasite sequences from the Asian flying fox genus *Cynocephalus* on the one hand and a second subgroup that obtains again two parasite sequences from the Asian flying fox genus *Cynocephalus* and the whole clade of African bat *Hepatocystis* parasites.

#### 3.2. Parasite blood stage morphology does not correspond to molecular data

*Hepatocystis* blood stages are confined to gametocytes, whereas schizont stages are limited to hepatocytes (Garnham, 1966). The majority of blood stages that were observed in the samples of the study (Giemsa-stained blood smears) were mature gametocytes. In a few samples, earlier stages (ring and immature gametocytes) occurred simultaneously with mature gametocytes. Ring stages exhibit large dense nuclei, resembling typical *Hepatocystis* ring stages, similar to parasites that infect African bats (Garnham, 1966). The characteristics of the gametocyte stages of the parasites of the study can be classified in two main groups. The first group (“category 1”, Table 1, Fig. 2 A–E) combines characteristics of descriptions of *Hepatocystis levinei* and *Hepatocystis pteropi* and was found in samples from *P. conspicillatus* and *P. scapulatus*. In one sample of *P. conspicillatus* (*P. conspicillatus*\_L20) further developed immature stages were found that occupy about a quarter of the erythrocyte and exhibit an elongate or pyriform shape, resembling the original description by Landau et al. (1985) of trophozoite stages of *H. levinei* (Fig. 2 C<sup>1+2</sup>). The (male) microgametocytes feature a nucleus that consists of a well-defined area of rounded dense pink chromatin, which is surrounded by a boundary of a lighter pink. In smaller microgametocytes, some pigment is overlapping the boundary, but in fully mature stages, this zone is free of pigment. The cytoplasm stains light blue. The pigment appears coarse and brown-greenish in colour (Fig. 2 A<sup>2</sup>, B<sup>2</sup>, C<sup>4</sup>). A few microgametocytes exhibited nuclei that were positioned towards the margin of the parasite cell. The characteristics, coarse pigment and nuclei that are placed marginally, comply with descriptions of *H. pteropi*, whereas the other resemble those of *H. levinei*. The (female) macrogametocytes generally featured a smaller nucleus than the microgametocytes, with a round to elongated form and pink colour. The cytoplasm stains a dark blue with pigment that was evenly distributed or sometimes more clustered around the nucleus area (Fig. 2 A<sup>1</sup>, B<sup>1</sup>, C<sup>3</sup>, D<sup>3</sup>).

The second group (“category 2”, Table 1, Fig. 2 F, G), found in samples of *P. alecto*, differed mainly in the appearance of the nucleus, both in micro- and macrogametocytes. The nucleus is diffuse and the nucleus area, which is free of pigment, appears rather grey. A diffuse nucleus was described in Landau et al. (2012a) for *Hepatocystis* sp. from *P. alecto* that could not be assigned to any known morphospecies.

Two exceptions to the aforementioned morphologies were apparent in two samples of *P. scapulatus* (*P. scapulatus*\_A4, Fig. 2 D; *P. scapulatus*\_A3, Fig. 2 E<sup>1+2</sup>), which featured gametocytes that resembled group one, but additionally exhibited a few gametocytes that shared characteristics with the descriptions of *Johnsprentia* (Landau et al., 2012b), thus possibly presenting a mixed infection. The mature microgametocytes stain intense pink to purple in colour, and feature a dense, darker-staining periphery (Fig. 2 E<sup>3+4</sup>). The pigment is distributed more towards the margin of the cell and the nucleus is elongated and peripheral, thus the peripheral regions appear denser than the centre. Co-infections of different haemosporidian genera have been reported from the host species *P. alecto*. The studies of Landau et al. (2012a, b) documented one species of the genus *Hepatocystis* and the newly described genera *Sprattiella* and *Johnsprentia* from the same host species *P. alecto* and stated that the three parasite genera co-occurred within individuals. However, the parasite sequences of the two *P. scapulatus* samples of the current study clearly grouped with *Hepatocystis*, and



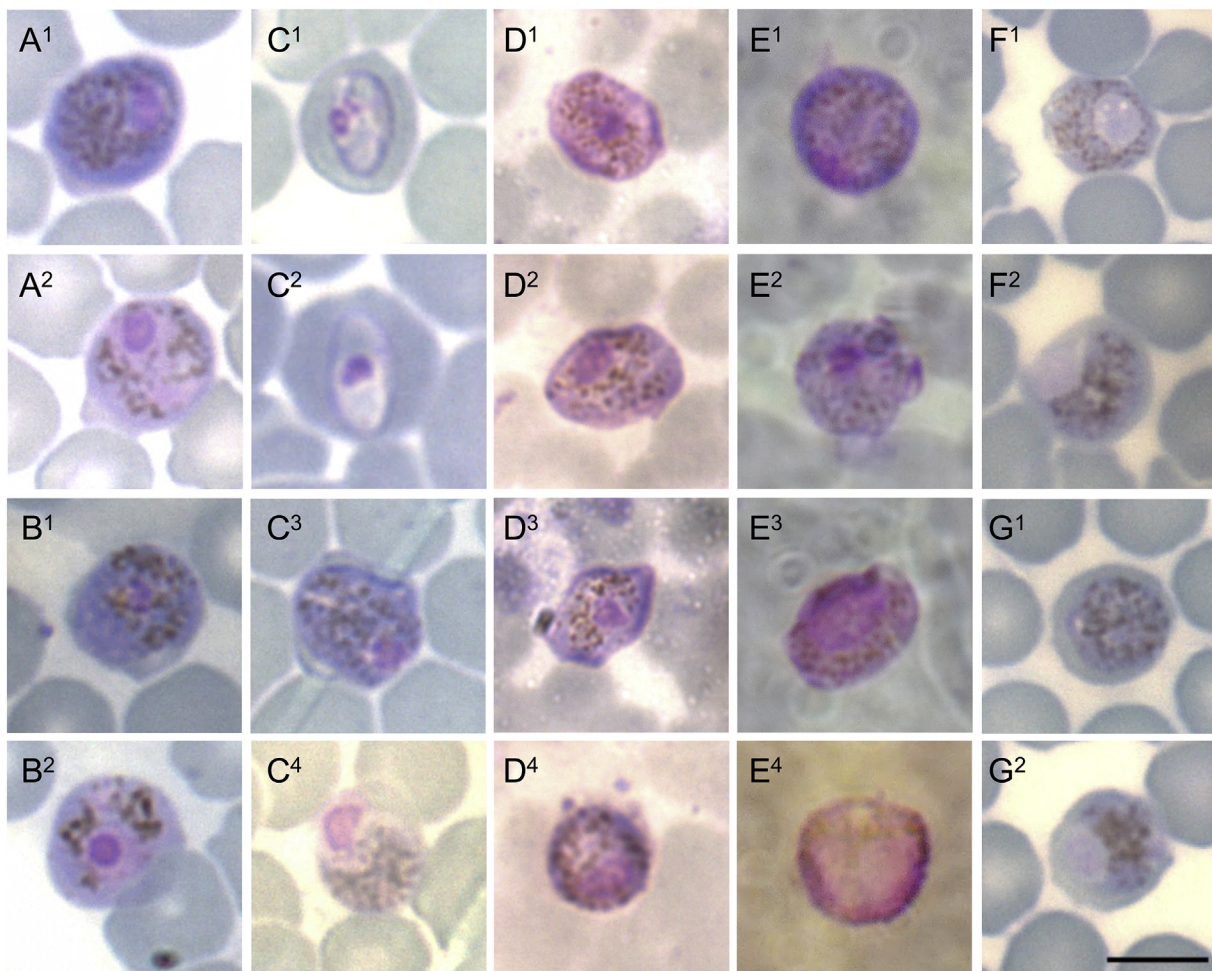
**Fig. 1.** Concatenated analysis. Bayesian analysis of concatenated alignment of two mitochondrial (*cytb*, *cox1*), one apicoplast (*clpC*), and two nuclear genes (*ef2*, *PAT*) rooted with *Leucocytozoon* species from birds. Posterior probabilities are given. **(A)** Clade of *Hepatocystis* presents the sister clade to mammalian *Plasmodium* (*Plasmodium*) and *Plasmodium* (*Vinckeia*) species. The parasite sequences of the study from Australian *Pteropus* species, form one distinct clade (together with three sequences from Asian *Pteropus* species) (highlighted in yellow). The sister clade contains all sequences from primate *Hepatocystis* (highlighted in blue), the African bat *Hepatocystis* parasites (highlighted in red) and sequences of *Hepatocystis* from Asian flying foxes of the genus *Cypropterus* and *Hipposideros*. **(B)** section from (A), uncollapsed *Hepatocystis* clades. Sequences of the study are highlighted in bold. Australasian *Hepatocystis* sequences from *Pteropus* hosts fall in two subclades and no host species specificity is apparent as the sequences from all three-host species group in two main clades. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

histological investigation of various tissues of these two animals did not demonstrate any haemsporidial schizonts. It cannot be ruled out though that a subpatent co-infection with *Johnspretia* was missed by our PCR approach, given that no molecular references for *Johnspretia* exist to date.

Together, haemsporidian parasites of the Australian *Pteropus* species *P. alecto*, *P. conspiciellatus* and *P. scapulatus* were identified as parasites of the genus *Hepatocystis* based on blood stage morphology and molecular phylogeny. The parasite sequences did not cluster according to host species, but grouped in two clades. Notably, both groups contained parasite sequences of parasites with different blood stage morphologies, which were from four to five *Pteropus* host species, respectively. We refrained from unambiguous assignments of the parasites to described *Hepatocystis* morphospecies, as the phylogenetic findings did not correspond to the morphology of the blood stages.

However, blood stage morphologies resembled *Hepatocystis* morphospecies that had been previously described from the investigated host species: *H. levinei* and *H. pteropi*, and an undetermined species of *Hepatocystis* (Manwell, 1946; Garnham, 1966; Landau et al. 1985, 2012a). The two “*Pteropus*”-specific parasite clades might comprise cryptic species or present two species complexes and the *Hepatocystis* morphospecies that have been described from *Pteropus* hosts might not present monophyletic species. Analyses of *Hepatocystis* parasites of additional *Pteropus* species are needed to confirm or reject the “*Pteropus*”-specific clade.

Interestingly, *Hepatocystis* parasites of the other flying fox genus *Cypropterus* seem to be more distantly related to the parasites from *Pteropus* hosts and show a closer relationship to parasites from primates and African bats. To resolve the overall *Hepatocystis* phylogeny, more complete taxon sampling from Asian bat hosts with sequences from



**Fig. 2.** Gametocyte blood stages of haemosporidian parasites of Australian bats. Giemsa-stained thin blood smears were investigated using oil immersion with a light microscope at a magnification of 1,000 $\times$ . **A)** ex *P. conspicillatus* (*P\_conspicillatus\_L2*, A<sup>1</sup> = macro-, A<sup>2</sup> = microgametocyte), **B)** ex *P. conspicillatus* (*P\_conspicillatus\_L15*, B<sup>1</sup> = macro-, B<sup>2</sup> = microgametocyte), **C)** ex *P. conspicillatus* (*P\_conspicillatus\_L20*, C<sup>1+2</sup> = early gametocyte stages, C<sup>3</sup> = macro-, C<sup>4</sup> = microgametocyte), **D)** ex *P. scapulatus* (*P\_scapulatus\_A4*, D<sup>1+2</sup> = micro-, D<sup>3+4</sup> = macrogametocytes), **E)** ex *P. scapulatus* (*P\_scapulatus\_A3*, E<sup>1+2</sup> = macrogametocytes, E<sup>3+4</sup> = unusual microgametocytes) **F)** ex *P. alecto* (*P\_alecto\_L8*, F<sup>1+2</sup> = microgametocytes) **G)** ex *P. alecto* (*P\_alecto\_L15*, G<sup>1</sup> = macrogametocyte, G<sup>2</sup> = microgametocyte), “ex” denotes that parasites were isolated from the respective host species. Bar = 5  $\mu$ m.

additional loci is needed. Cytochrome *b* still remains the most used phylogenetic marker in haemosporidian phylogenies and therefore available sequences for primate *Hepatocystis* parasites as well as for almost all Asian bat *Hepatocystis* parasites are limited to cytochrome *b*.

Together our findings underline the important role of bats in the evolutionary history of malaria parasites and the new molecular data revealed, once again, unexpected phylogenetic relationships among the haemosporidian parasites.

#### Declarations of interest

None.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ijppaw.2018.06.001>.

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