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Short communication

Molecular investigation of *Hepatocystis* parasites in the Australian flying fox *Pteropus poliocephalus* across its distribution range[☆]

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ABSTRACT

Phylogenetic inference of *Hepatocystis*, a haemosporidian parasite of diverse primate and bat hosts, revealed that the parasites from Australasian *Pteropus* bat species form a distinct clade to all other *Hepatocystis* parasites from Africa and Asia. Here, we investigate the phylogenetic placement of *Hepatocystis* in the Australian bat *Pteropus poliocephalus* for the first time and examine parasite morphology and prevalence from selected points across its range. *Hepatocystis* infections were detected in low prevalences in *P. poliocephalus* in contrast to high numbers in *P. alecto* and *P. scapulatus.* The prevalence in *P. poliocephalus* varied across its distribution range with 15% in the central biogeographic areas (central Queensland and New South Wales) and 1% in the southern-most edge (South Australia) of its range. Sequencing of five genes revealed high genetic similarity in *Hepatocystis* of *P. poliocephalus* independent of sampling location. Phylogenetic analysis placed these parasites with *Hepatocystis* from other *Pteropus* species from Australia and Asia. While numerous haplotypes were identified among sequences from the *Pteropus* hosts, no patterns of host specificity were recovered within the *Pteropus*-specific parasite group.

1. Introduction

Malaria parasites belong to a diverse group of over 500 haemosporidian parasite species (Haemosporida) that infect both dipteran invertebrate and vertebrate host groups (Martinsen and Perkins, 2013). Ten different haemosporidian parasite genera are described from mammals, including the closely related genera *Plasmodium* and *Hepatocystis* (Perkins and Schaer, 2016). *Hepatocystis* parasites commonly infect non-human primates and bats in Africa and Asia (e.g. Boundenga et al., 2018; Garnham, 1966), and they have also been described from the four Australian flying fox (fruit bat) species, *Pteropus alecto* (Black Flying Fox)*, Pteropus conspicillatus* (Spectacled Flying Fox)*, Pteropus poliocephalus* (Grey-headed Flying Fox) and *Pteropus scapulatus* (Little Red Flying Fox) (e.g. Breinl et al., 1912; Landau et al., 1985), but parasite prevalence in the four hosts remains unknown. Recently, a phylogenetic analysis of *Hepatocystis* parasites from *P. alecto*, *P. conspicillatus* and *P. scapulatus* revealed that these parasites form a *Pteropus*-specific group together with parasites of Asian *Pteropus* species (Schaer et al., 2018). *Hepatocystis* parasites of the fourth Australian flying fox species, *P. poliocephalus,* have not been analyzed by molecular genotyping to date. Morphological studies identified *P. poliocephalus* as host for two *Hepatocystis* species*, Hepatocystis pteropi* (Mackerras, 1959) and *Hepatocystis levinei* (Landau et al., 1985). Here we investigate the occurrence of *Hepatocystis* parasites in *P. poliocephalus* from selected areas across its distribution range in Australia with a focus on the southern-most colony (Adelaide, South Australia). The phylogenetic relationships of *Hepatocystis* from *P. poliocephalus* among those of the other three Australian *Pteropus* species were investigated.

[☆] Note: Nucleotide sequence data reported in this paper are available in the GenBank database under accession nos. MK640503-MK640552 and MN136863-MN136913.

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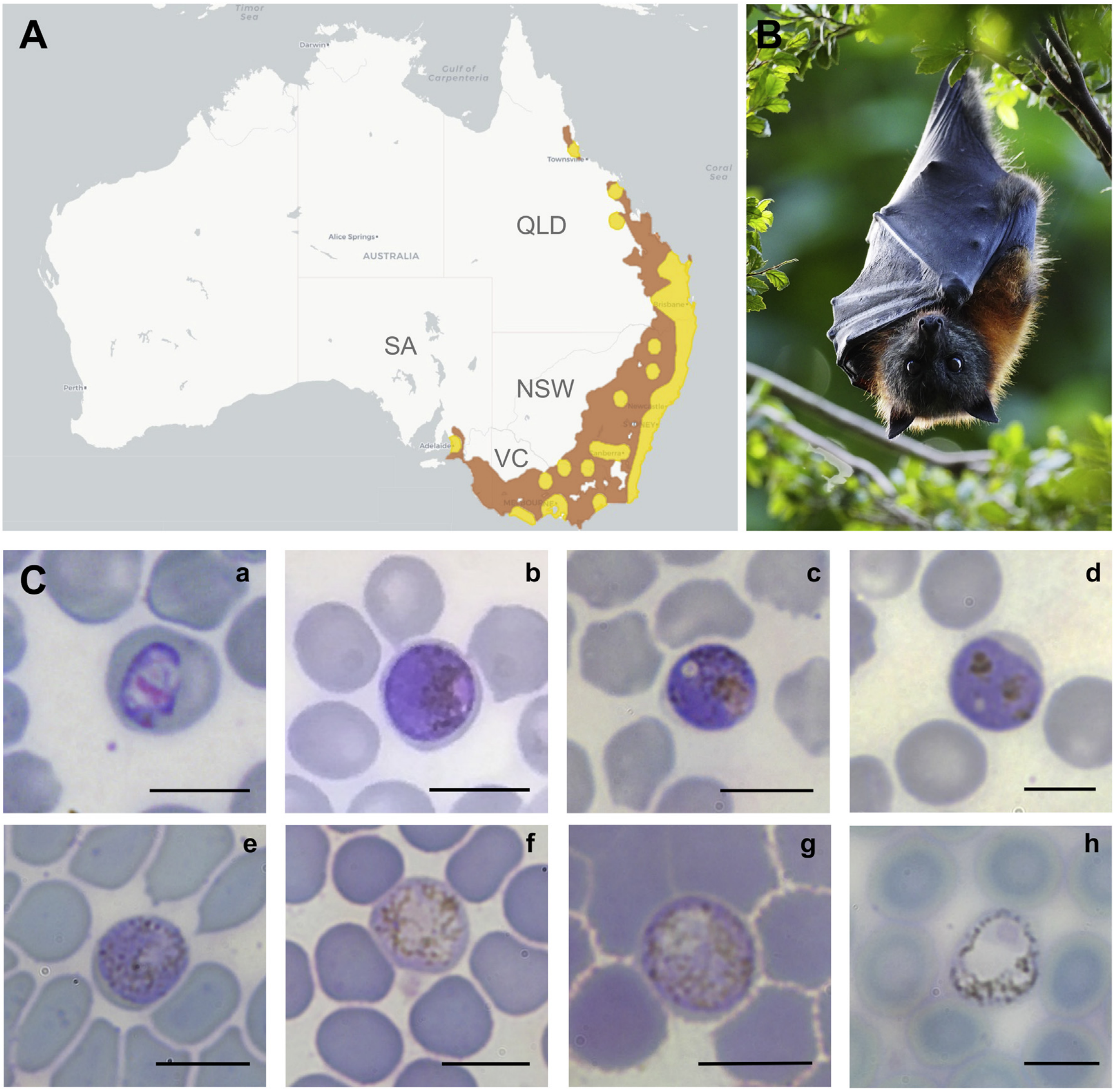

Fig. 1. *Hepatocystis* infections in *Pteropus poliocephalus*. A) Distribution range of *Pteropus poliocephalus. P. poliocephalus* is distributed across Southern Queensland (QLD), New South Wales (NSW), Victoria (VIC) and in 2010 this species established a permanent colony in Adelaide, South Australia (SA) (naturalresources.sa.gov.au). The map shows the GIS data of areas, where *P. poliocephalus* or species habitat is likely to occur (highlighted in yellow) or may occur (highlighted in brown). The map was created with the software Mango (https://mangomap.com) with species distribution data (www.environment.gov.au). B) Adult *P. poliocephalus* individual from Wingham Brush, New South Wales (picture by AM). C) Giemsa-stained thin blood smears at a magnification of 1000 ×. Depicted are gametocyte blood stages of *Hepatocystis* parasites of *P. poliocephalus* (1000 ×). First row: ex *P. poliocephalus,* 1026, Adelaide: a) immature stage with elongate or pyriform shape; b, c, d) mature macrogametocytes. Second row: ex *P. poliocephalus,* 1280, Adelaide: f, h) microgametocytes; e, g) macrogametocytes. "ex" denotes that parasites were isolated from the respective host species. Bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Methods

We sampled three Australian flying-fox species (*Pteropus alecto, P. poliocephalus, P. scapulatus*) for this study. Species identification was aided by Churchill (2008). *Pteropus alecto* and *P. scapulatus* range from Western Australia (WA), Northern Territory (NT), Queensland (QLD) and New South Wales (NSW) (Field et al., 2012). In contrast, *P. poliocephalus* is primarily distributed across Southern QLD, NSW and Victoria (VIC) (Fig. 1A). In 2010 *P. poliocephalus* established a permanent colony in Adelaide, South Australia (SA) and the colony has steadily increased in number (naturalresources.sa.gov.au). Of note, the four *Pteropus* species occur in sympatry in parts of their distributions, with mixed species colonies in some areas (Parsons et al., 2010).

Samples from *P. poliocephalus* were acquired from QLD, NSW and SA to capture the north-south extensions of their current distribution range (Fig. 1A, B) and from *P. alecto* and *P. scapulatus* from QLD between 2015 and 2018 (Table 1). Blood (0.5 ml) was collected from the brachial or propatagial vein of anaesthetized (Isoflurane and oxygen) bats and spotted onto DNA FTA cards (Whatman Australia) for molecular analyses and thin blood smears prepared for microscopy. Morphological identifications of the parasites were done by microscopic examination of Giemsa-stained thin blood smears at a magnification of 1000 ×. Liver samples of *P. poliocephalus* from NSW were collected aseptically during post-mortems of bats that had died from injury or heat stress. Capture and sampling of bats was approved by institutional Animal Ethics Committees (Macquarie University ARA2017/013 Power, the University of Adelaide No. S-2015-028, Boardman and CSIRO No. 2016–17, Westcott) and Scientific permits from SA (M-23671-1,2 and 3) and QLD (173(P)) and NSW (# SL101898 & SL101050).

Haemosporidian parasites were detected and identified using microscopy and molecular methods (Schaer et al., 2013, 2018). Genomic DNA was extracted from DNA FTA spots and liver samples using the

Table 1
Investigated bats of the study. Listed are species, collection date, site and prevalences of *Hepatocystis* infections.

Host species	Locality, city (state*)	Date	Total #	# of inf. individuals	Prevalence in %
P. alecto		**All**	**29**	**13**	**45**
	Northern, Burketown (QLD)	Apr 2018	5	2	40
	Northern, Coen (QLD)	Apr 2018	8	5	63
	Northern, Charters (QLD)	Jul-Aug 2018	5	2	67
	Central, Canungra (QLD)	Apr 2015	2	0	0
	Central, Gympie (QLD)	Apr 2015	7	3	43
	Central, Woolgoolga (NSW)	Apr 2015	2	1	50
P. scapulatus		**All**	**6**	**6**	**100**
	Northern, Charters (QLD)	Jul-Aug 2018	5	5	100
	Central, Woolgoolga (NSW)	Apr 2015	1	1	100
P. poliocephalus		**All**	**258**	**8**	**3**
	Central, Gympie (QLD)	Apr 2015	2	0	0
	Central, Canungra (QLD)	Apr 2015	9	1	11
	Central, Woolgoolga (NSW)	Apr 2015	12	2	17
	Central, Sydney (NSW)	Mar-Apr 2018	8	3	38
	Central, Sydney (NSW)	Sept 2018	10	0	0
	Southern, Adelaide (SA)	Sep 2015	38	0	0
	Southern, Adelaide (SA)	Feb 2016	32	0	0
	Southern, Adelaide (SA)	Aug 2016	20	0	0
	Southern, Adelaide (SA)	Feb 2017	21	0	0
	Southern, Adelaide (SA)	Sept 2017	21	0	0
	Southern, Adelaide (SA)	Feb 2018	44	1	2
	Southern, Adelaide (SA)	July 2018	41	1	2

* QLD (Queensland), NSW (New South Wales), SA (South Australia), Sampling of *P. poliocephalus* in SA was undertaken twice per year from 2015 to 2018 and QLD *Pteropus* species were sampled once in either 2015 or 2018.

Qiagen DNeasy blood and tissue extraction kit (Hilden, Germany). PCR amplification of two mitochondrial genes (*cytb*, *cox1*), one apicoplast (*clpC*) and two nuclear genes (*EF2, PAT*) (Table S1) and bi-directional sequencing were performed (BigDye v3.0 Applied Biosystems) and run on an ABI-373 sequencer. Sequences were edited and aligned in Geneious vs.8. Phylogenetic analysis was performed to infer relationships between *Hepatocystis* parasites from *P. poliocephalus* to those previously described from other *Pteropus* species (Schaer et al., 2018) (Table S2). Sequences were concatenated and data divided into partitions according to the five genes. The concatenated alignment comprised a total length of 3662 bp (including 906 bp of *cytb*, 930 bp of *cox1*, 528 bp of *clpC*, 516 bp of *EF2* and 782 bp of *PAT*). DNA substitution models and partition schemes were evaluated with PartitionFinder vs.2 (Lanfear et al., 2017) and Bayesian inference, using *Leucocytozoon* as outgroup, was conducted in MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001), via the CIPRES Science Gateway Web Portal V3.3 (Miller et al., 2010) with two runs of four chains (three heated, one cold, temperature = 0.01) each for 25 million generations and a burn-in of the first 25% trees. Mixing and convergence of runs and effective sample size (ESS $>$ 300) were evaluated in Tracer v1.6 (Rambaut et al., 2014).

3. Results and discussion

In total, 293 bats comprising *P. poliocephalus* (n = 258), *P. alecto* (n = 29) and *P. scapulatus* (n = 6) were screened for haemosporidian infections, with *Hepatocystis* parasites detected in all three species (Table 1, Table S3). For *P. poliocephalus*, the overall prevalence was 3% (8/258) in contrast to prevalences in *P. alecto* (45%, 13/29) and *P. scapulatus* (100%, 6/6) (Table 1). The prevalence in *P. poliocephalus* varied across its distribution range with 15% (6/41) in the central biogeographic area (QLD, NSW) and 1% (2/217) in the Southern area (SA). The two infected individuals in the SA colony were sampled in 2018. The low prevalence and onset of infections with *Hepatocystis* parasites in *P. poliocephalus* in SA in 2018 are intriguing. The SA colony has been sampled biannually since September 2015 as part of a disease surveillance study, but no haemosporidian infections were previously detected in 132 samples. Several factors may be contributing to this finding. First, *Hepatocystis* parasite transmission might not occur in the SA colony, either because the dipteran vector(s), probably *Culicoides* species (Landau et al., 1985), is missing or because the founding *P. poliocephalus* population lacked a parasite reservoir. A plausible explanation is that the two infected individuals joined the colony only very recently. Long distance dispersal has been reported from *P. poliocephalus*, with movements of up to 500 km over 48 h (Roberts et al., 2012). While numbers of bats in the SA colony has steadily increased since 2010, their numbers increased from approximately 17,000 to 27,000 in June 2018 (J van Weenan, pers. comm.). In an alternative scenario, parasite transmission is possible in the SA colony, and the recent onset of rare infections can be attributed to the distinct climate, which contrasts with the tropical and subtropical climate zones of the coastal areas of QLD. SA features the hottest and driest conditions within the Australian distribution range of *P. poliocephalus.* In QLD, where all four *Pteropus* species display abundant *Hepatocystis* infections, tropical conditions might be superior for *Hepatocystis* transmission in Australia. To what extent average temperature and seasonal fluctuations affect *Hepatocystis* sporogony, as well as vector abundance and transmission capacity remains enigmatic. Another explanation might be that few settling individuals of the founding population were infected and efficient transmission with the local vector population is only established over time. Further sampling across the flying-fox distribution range is needed to clarify the drivers of prevalence, and the identity of the dipteran vector of chiropteran *Hepatocystis* parasites in Australia needs to be ascertained.

The morphology of the gametocyte blood stages of *P. poliocephalus* features characteristics of the morphospecies *H. levinei, H. pteropi* and *Hepatocystis* sp. (Garnham, 1966; Landau et al., 1985, 2012) (Fig. 1C), similar to infections in the other Australian *Pteropus* hosts (Schaer et al., 2018). Exemplarily, immature gametocyte stages that exhibit elongate or pyriform shapes resemble descriptions of *H. levinei* (Fig. 1C, a). Mature gametocytes with dark blue staining cytoplasm and hemozoin pigment clustered around the nucleus area (Fig. 1C, b) or evenly distributed (Fig. 1C, c) could be attributed to *H. levinei* and *H. pteropi.* Further, the diffuse appearance of the nuclei of micro- (Fig. 1C, f,h) and macrogametocytes (Fig. 1C, e,g) were described from *Hepatocystis* sp. of *P. alecto* (Landau et al., 2012).

This first molecular analysis of the *Hepatocystis* parasites of *P. poliocephalus* revealed a close relationship with *Hepatocystis* parasites of *P. alecto, P. conspicillatus, P. scapulatus* and Asian *Pteropus* species

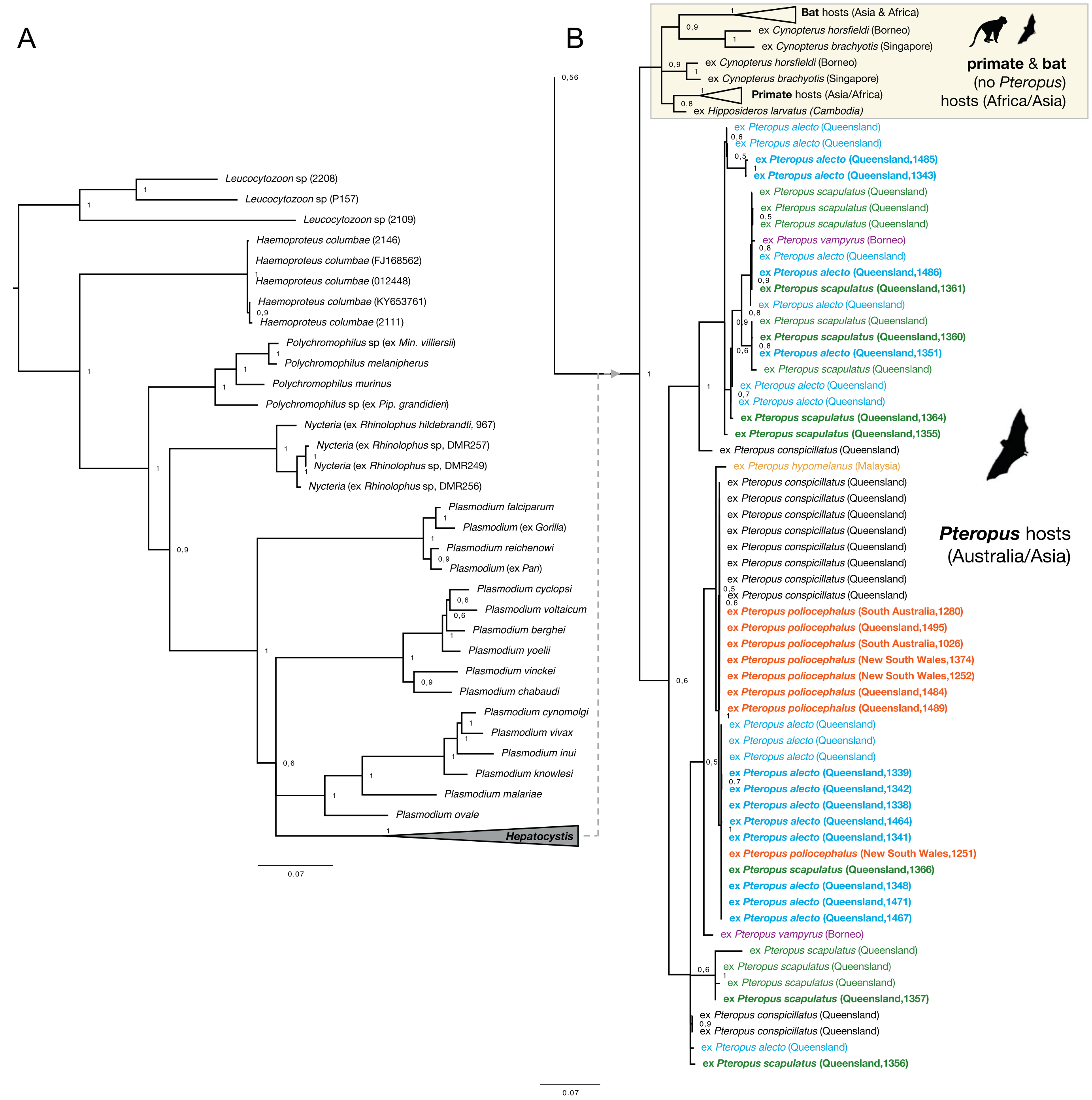

Fig. 2. Bayesian analysis of concatenated alignment of two mitochondrial genes (Cyt*b*, Cox1), one apicoplast (clpC) and two nuclear genes (EF2, PAT) rooted with *Leucocytozoon* species from birds. Posterior probabilities are given. A) *Hepatocystis* as collapsed clade groups with the *Plasmodium (Vinckeia)* and *Plasmodium (Plasmodium)* species. B) Uncollapsed *Hepatocystis* clade. *Hepatocystis* parasite sequences of African bat species (small collapsed clade) and of African and Asian primate species (small collapsed clade) group with *Hepatocystis* parasite sequences of Asian non-*Pteropus* hosts. All *Hepatocystis* parasites from *Pteropus* hosts group basal to the aforementioned clades. All sequences from this study are in bold text, parasite sequences from *P. poliocephalus* are highlighted in red. The parasite sequences of *P. poliocephalus* as well as the new samples from *P. alecto* (highlighted in blue) and *P. scapulatus* (highlighted in green) group among the published *Hepatocystis* sequences from Australian and Asian *Pteropus* species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(accession numbers MK640503-MK640552, MN136863-MN136913) (Fig. 2). There is neither an apparent geographic pattern nor signatures of host specificity among *Hepatocystis* parasites in Australian flying-foxes. Diverse haplotypes are present, which we interpret as a *Hepatocystis* species-complex in *Pteropus* bats. Accordingly, assignment to one morphospecies is not possible as the morphology of the blood stages differ between different samples of *P. poliocephalus* despite identical nucleotide sequences (e.g. parasites ex *P. poliocephalus,* samples 1026 and 1280 from Adelaide, Figs. 1C and 2). This finding raises the intriguing question of plasticity of gametocyte morphology in response to host factors, such as age, immunity and nutritional status, which might not permit species assignments based on gametocyte morphology.

However, large sampling efforts and investigation of blood and tissue stages concurrent with molecular analysis are needed to unambiguously link morphological and molecular data to the corresponding *Hepatocystis* species. Our analysis identified all *Hepatocystis* parasites of *Pteropus* as basal to a clade comprising parasites of African and Asian primate and non-*Pteropus* bat hosts, which agrees with our previous findings (Schaer et al., 2018). This study shows that *Hepatocystis* parasites of all four Australian mainland *Pteropus* species are closely related, resulting in an apparent lack of host specificity or geographic pattern. *P. poliocephalus* bats are infected with *Hepatocystis* parasites that feature low genetic diversity across the north-south extension of their distribution range in Australia, encompassing both tropical and temperate zones. A low *Hepatocystis* prevalence in the southern-most *P. poliocephalus* colony remains unexplained but raises the possibility of differences in parasite susceptibility and/or vector density that may be due to differing climatic conditions influencing parasite transmission dynamics. This study illustrates the necessity for large sampling across the entire distribution range in parasitic infections that feature low prevalences.

Declaration of Competing Interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103978.

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