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# *Hepatozoon* Parasites (Apicomplexa: Adeleorina) in Bats

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ABSTRACT: We provide the first evidence of *Hepatozoon* parasites infecting bats. We sequenced a short fragment of the 18S rRNA gene (~600 base pairs) of *Hepatozoon* parasites from 3 *Hipposideros cervinus* bats from Borneo. Phylogenies inferred by model-based methods place these *Hepatozoon* within a clade formed by parasites of reptiles, rodents, and marsupials. We discuss the scenario that bats might be common hosts of *Hepatozoon*.

The protozoan genus *Hepatozoon* is comprised of intracellular parasites of tetrapods and hematophagous arthropods that serve as definitive hosts. Transmission of parasites to tetrapods is thought to be mainly by ingestion of infected prey, most commonly arthropods (Smith, 1996). Species of *Hepatozoon* have been recovered from all taxonomic classes of tetrapods, and within the class Mammalia there are records of species parasitizing members of the following orders: Dasyuromorphia, Didelphimorphia, Diprotodontia, Hyracoidea, Microbiotheria, Rodentia, Lagomorpha, Carnivora, Peramelemorphia, and Soricomorpha (Smith, 1996; Merino et al., 2009). Nonetheless, to the best of our acknowledge there are no published records of *Hepatozoon* parasitizing bats, order Chiroptera, the second-most speciose order of mammals, and potentially the order with the highest number of species with an insectivorous diet (Nowak, 1991). In this study, we demonstrate for the first time bats infected by *Hepatozoon* sp., and we provide a phylogenetic hypothesis on the placement of this parasite species.

During a molecular survey of trypanosomes in mammalian tissues, we tested different sets of primers for amplification and sequencing of *Trypanosoma* spp. on 7 DNA samples extracted from liver of *Hipposideros cervinus* bats collected 30 January 2007 in rain forest at Bukit Sarang on the Ulu Kakus in Sarawak, Malaysian Borneo. For 1 set of those primers, we accidentally amplified *Hepatozoon* rather than *Trypanosoma* DNA. We conducted polymerase chain reactions (PCRs) with illustra puReTaq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.) to amplify a fragment of the 18S rRNA gene using the primers SS4_F (GTGCCAGCACCCGCGGTAAT) and the 18Sq2R (AAAGCGGCCATGCACCACCA) and the touchdown PCR profile described by Murphy and O'Brien (2007), with a modification allowing denaturation at 95 C for 5 min. We obtained an amplification of a product of ~700 base pairs (bp) instead of the ~950-bp product expected from *Trypanosoma* spp. We cleaned the PCR products with ExoSAP-IT (Affymetrix Inc., Santa Clara, California) and conducted sequencing reactions with the ABI BigDye chemistry (Applied Biosystems, Inc., Foster City, California). We sequenced the products on an ABI 3730xl DNA Analyzer automatic sequencer (Applied Biosystems, Inc.).

We used BLAST comparisons to pinpoint the identity of our newly generated DNA sequences as *Hepatozoon*. Later, we built a matrix comprising our DNA sequences together with selected *Hepatozoon* sequences from other studies (Carreno et al., 1999; Criado-Fornelio et al., 2006, 2009; Sloboda et al., 2007; Merino et al., 2009; Harris et al., 2011; Maia et al., 2011; Barta et al., 2012). To align the sequences we used the Consensus Align tool in Geneious Pro version 5.6.2 with default parameters (Drummond et al., 2010). The alignment was checked manually for obvious misplacements. The matrix comprised 52 terminals by 1,730 bp after alignment; with sequences ranging from 604 bp to 1,679 bp. We conducted phylogenetic analyses using maximum likelihood (ML) and Bayesian inference (BI). For both methods we used the HKY+I+G model of sequence evolution, as it was indicated by jModelTest, using the Bayesian information criterion (Posada, 2008) to have the best fit to the data. We performed an ML analysis in PhyML 3.0 (Guindon et al., 2010) using 5 neighbor-joining trees as random starting trees and the SPR algorithm for tree improvements, and we estimated node supports by 1,000 bootstrap pseudo-replicates. We conducted the BI analyses in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) in 2 independent runs with 4 cold and 2 heated chains, sampling 100 trees every 1,000 generations. The analysis was allowed to run until reaching convergence of the runs (convergence diagnostic value, stopval, set at 0.01); this convergence occurred at 1,211,000 generations, and the first 10% of the trees were discarded as burn-in. Chain convergence also was verified with the Tracer version 1.5 program (Drummond and Rambaut, 2007). Node support was determined by Bayesian posterior probabilities.

Of the 7 samples used, 4 amplified a fragment of ~700 bp. We successfully sequenced 3 of the 4 amplifications, and we used 607 bp in the phylogenetic analysis after trimming poor-quality ends and primer sequences. BLAST searches of our query sequences returned results for several *Hepatozoon* sequences that were nearly identical, including *Hepatozoon* sp. Pty01po (HQ734790), *Hepatozoon* sp. 3126tm (HQ734806), and *Hepatozoon ayorgbor* (EF157822). In the phylogenetic analyses, these sequences did not form a monophyletic group but clustered together in the clade including rodent and marsupial *Hepatozoon* species. Our phylogeny shows 2 clades in *Hepatozoon* (although neither is well supported); 1 clade is mostly comprised of the species parasitizing carnivores and reptiles, and the other clade includes the rodent, marsupial, bat, amphibian, and other reptile parasites. Both phylogenetic methods gave congruent topologies, with minimal differences in the nodal support (Fig. 1).

Here, we report genetic evidence that *Hepatozoon* may be infecting South East Asian bats (*Hipposideros cervinus*), evidence that would constitute the first time that any bat has been found harboring *Hepatozoon*. There is only a single morphological report of a potential *Hepatozoon* infecting a bat; however, no definitive identification has been provided (Lainson and Naiff, 2000). DNA sequences presented in this paper might be viewed as inconclusive evidence of sustained infections of *Hepatozoon* parasites in bats until gamonts can be detected by microscopy (e.g., Valkiūnas et al., 2011); therefore, we recommend future efforts to collect blood smears from hosts in addition to preserved blood and tissue samples.

Our sequences cluster in the clade comprised of rodent, marsupial, amphibian, and reptile parasites, a clade that has a higher diversity of hosts, and perhaps more complex life cycles, than the clade formed by the *Hepatozoon* parasites of carnivores + reptiles. In general, the phylogeny is fairly similar to previously published trees (Harris et al., 2011; Maia et al., 2011; Barta et al., 2012) and does not show any apparent patterns of either host–parasite coevolution or biogeographic association (Fig. 1). This lack

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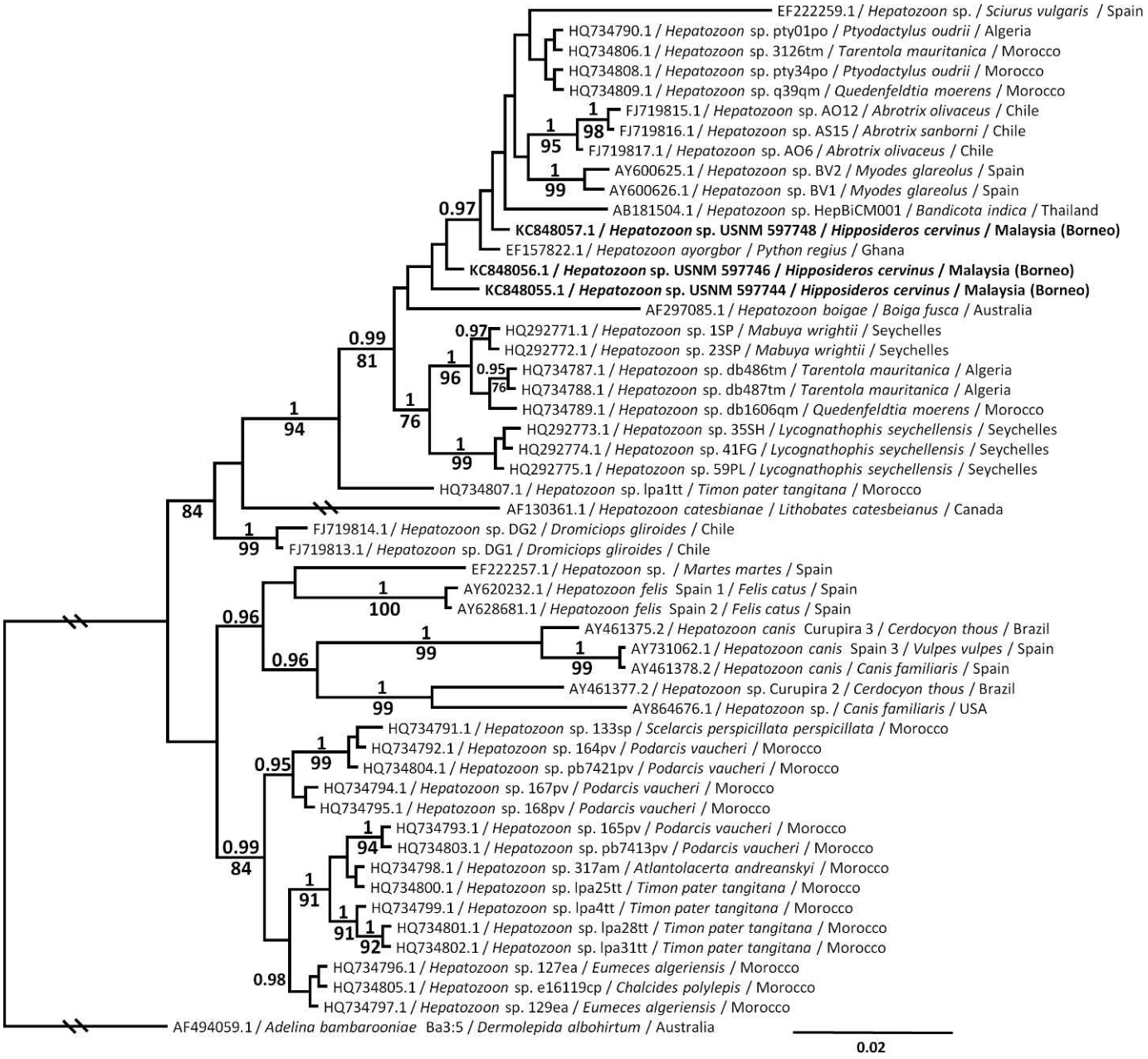


FIGURE 1. Bayesian Inference phylogram depicting the relationships among DNA sequences of the18S rRNA gene of *Hepatozoon*. Numbers above the branches correspond to the BI posterior probabilities, and the numbers below the branches are the ML bootstrap scores. Only values >0.95 for BI and >75 for ML are indicated. Terminal labels contain the following information: 18S rRNA NCBI accession number/parasite name and code/host/place of origin. The *Hipposideros* specimens are vouchered in the collections of the National Museum of Natural History, Smithsonian Institution (USNM).

of pattern, at least at biogeographic scales, might be a result of incomplete taxon sampling of parasites (Lecointre et al., 1993) or the use of the 18S rRNA gene as the marker for inferring relationships because this gene may not be providing an accurate tree (e.g., Pinto et al., 2012). Patterns may likely emerge after extensive molecular surveys of *Hepatozoon* parasites using multiple genetic markers. In addition to continued collecting efforts, molecular surveys of *Hepatozoon* could be facilitated by surveying museum tissue collections (e.g., Pinto et al., 2010).

Bats could be effective hosts of *Hepatozoon* parasites because of the exclusive insectivorous diet of several families of the order, and several of the fruit- and nectar-feeding species are also facultative insectivores (Nowak, 1991; Dumont et al., 2012). The ability to ingest infected arthropods may facilitate contaminative infection, a main mode of *Hepatozoon* transmission among vertebrates (Smith, 1996). In addition, bats are among the most social mammals (Kerth, 2008), and grooming practices may increase the chance of *Hepatozoon* transmission by ingesting infected ectoparasites. Alternatively, several species of bats depredate small vertebrates and thus may well be another mode of transmission, as discussed for mammals of the order Carnivora (Desser, 1990; Baneth and Shkap, 2003; Johnson et al., 2009; Allen et al., 2011).

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