Title: First report of a novel *Hepatozoon* sp. identified in giant pandas (*Ailuropoda melanoleuca*)

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Running Head: Novel *Hepatozoon* sp. identified in giant pandas

Key Words: *Hepatozoon*, *Ailuropoda melanoleuca*, giant panda, apicomplexa, hemoparasite, conservation management, polymerase chain reaction

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Abstract:

The first report of giant pandas (*Ailuropoda melanoleuca*) infected with a novel *Hepatozoon* species is presented. An intraleukocytic parasite was detected via routine blood smear from a zoo-housed giant panda. Ribosomal DNA sequences indicated a previously undescribed *Hepatozoon* species. Phylogenetic and distance analyses of the sequences placed it within its own branch, clustered with Old World species with carnivore (primarily ursid and

mustelid) hosts. Retrospective and opportunistic testing of other individuals produced additional positive detections (17/23, 73.9%), demonstrating 100% prevalence (14/14) across five institutions. All animals were asymptomatic at time of sampling, and health implications for giant pandas remain unknown.

Hepatozoon spp. are apicomplexan hemoparasites capable of infecting a wide range of vertebrate taxa globally, including canids, ursids, felids, and others (André et al., 2010; East et al., 2008; Kubo et al., 2006; Kubo et al., 2008; Kubo et al., 2010; Pawar et al., 2011; Pawar et al., 2012). In North America, *Hepatozoon* infections have been documented in domestic canids, coyotes (*Canis latrans*), and other carnivores (Kocan et al., 2000; Mercer et al., 1988). Clinical disease is uncommon but can cause musculoskeletal lesions and potentially death (Baneth et al., 2003). *Hepatozoon* spp. have heteroxenous life cycles, requiring definitive invertebrate hosts and intermediate vertebrate hosts (Smith, 1996). Unlike the bite-transmission route characteristic of other vector-borne pathogens, *Hepatozoon* spp. are typically transmitted via host ingestion of the arthropod during grooming or consumption of infected prey or prey with infected ticks (Allen et al., 2011; Baneth et al., 2003; Smith, 1996).

This report describes a novel *Hepatozoon* parasite found in zoo-housed giant pandas (*Ailuropoda melanoleuca*). Giant pandas are ursids native to central China and an iconic flagship species for conservation. Currently categorized as Vulnerable by the IUCN, the species faces continued threats from habitat loss and fragmentation, starvation, and infectious diseases (Feng et al., 2015; Swaisgood et al., 2016; Zhang et al., 2008).

In March 2005, an intraleukocytic hemoparasite was detected on a routine blood smear of a male giant panda housed at the Smithsonian National Zoological Park (NZP) in Washington, DC (listed as GP4 in Table 1). The blood smear demonstrated neutrophil-associated parasites with a morphology similar to that of known *Hepatozoon* species. The specimen could not be identified to species by light microscopy.

DNA was extracted from the sample using DNeasy kits (Qiagen) and amplified via polymerase chain reaction (PCR) using a combination of 18S ribosomal DNA primer sets

(Hep18S2-H, Hep18S4-L, Hep18S4-H, BT1-L, BT1-H, BTH1-L, and BTH1-H) in a 25 μl PCR. Sequencing was conducted on an ABI 3730, and sequences were aligned and edited using Sequencher 4.1. A total length of 1092 bp of 18S sequence was obtained for GP4. His mate, GP5 (MeiXiang, 113607), was also positive, and we obtained 1111 bp of sequence for her which was identical to that of GP4. The latter sequence was utilized in subsequent analyses. Blasts to GenBank sequences from morphologically identified specimens and phylogenetic analyses confirmed identification of the parasite lineage to genus (*Hepatozoon*).

The program RAxML 8.2.11 (Stamatakis, 2014) implemented in Geneious (Kearse et al., 2012) was used to estimate phylogenetic relationships within genera using a maximum likelihood (ML) criterion and a general time reversible (GTR+I+gamma) model of nucleotide substitution, with 1000 bootstrap replications. jModelTest (Darriba et al., 2012; Guindon and Gascuel, 2003) was used to identify the best evolutionary model for the sequence data. Under BIC and DT (decision theory) criteria, the GTR + I + G model was preferred, while the AICc criterion favored the slightly different TPM1uf+I+G model. The estimated I and G values in all models were nearly identical (0.49 and 0.89, respectively). Therefore we selected the GTR + I + G model from the BIC for the ML analysis.

In the resulting phylogeny, the giant panda-associated *Hepatozoon* lineage occupied its own distinct, long branch, nested in a clade with Old World species *H. felis*, *H. ursi*, and *H. martis* (Figure 1). The consensus phylogeny suggested a genetically unique parasite, most closely related to a sister clade containing *H. ursi*, a parasite of Asiatic bears (Kubo et al., 2008; Pawar et al., 2011), and *H. martis*, a mustelid parasite (Hodžića et al. 2018), but bootstrap support of 52% was marginal for this node, and it essentially collapses to a trichotomy. The giant panda *Hepatozoon* 18S sequence was 3.2 to 3.6% divergent (uncorrected) from those of *H. ursi*

and *H. martis*, but ≥4.5 % divergent from all other carnivore *Hepatozoon* sequences. Thus, we found support for a single clade containing ursid (including panda) and mustelid *Hepatozoon* derived sequences. We also found two paraphyletic *H. felis* clades, with one falling out as a poorly supported sister clade to *H. americanum*. However, analyses with additional *H. felis* sequences sometimes removed the paraphyly and the two *H. felis* clades became poorly supported sister clades. Regardless, the trees and data support that the giant panda *Hepatozoon* is a distinct lineage and species, and that it is most closely related to *Hepatozoons* with ursid and mustelid hosts.

Conventional PCR was performed on 23 archived or opportunistically collected blood and tissue samples from 14 giant pandas between 1982—2006. Primer sets BT1 (432 bp amplicon) and BTH1 (751 bp amplicon) provided the greatest amplification and sequencing consistency and were tested on all available samples. For most individuals, up to 1113 bp were obtained. Whole blood samples were evaluated where possible, but when unavailable, other tissue types, plasma, or stained blood smears were substituted. For six pandas, multiple samples were used. The samples had been collected from seven adult males, six adult females, and one male neonate from five institutions (three in the United States, one in the United Kingdom, and one in China). All individuals were born in China (either wild-caught or captive-bred) with the exception of the neonate, which was born at the NZP but did not survive beyond a few days.

The PCR results from testing 14 captive giant pandas are summarized in Table 1. All individuals (14/14, 100%) sampled demonstrated positive tests for *Hepatozoon* sp., identical in overlapping sequence to that of GP4 and GP5. Positive detections were made in 17 samples out of 23 tested (73.9%). Of the negative results, four resulted from stained blood smears and two from whole blood samples. These were presumed to be false negatives, attributed to poor quality

of archival samples and varying extraction efficiency and PCR sensitivity due to DNA inhibitors (Scopel et al., 2004; Shavey and Morado, 2012). The sequence for giant panda 113607 was deposited in GenBank (accession number MK645858).

Although the level of parasitemia was not quantified, infrequent findings on routine microscopy and occasional negative results by PCR suggest that the parasite may be present at low levels or intermittently in circulation (Otranto et al., 2011; Scopel et al., 2004). Challenges in detecting the parasite without molecular methods may explain why the parasite was not previously found. It is possible that the parasite is abundant in other tissues, as these may be sites of merogony and cyst formation in mammalian *Hepatozoon* life cycles (Smith, 1996), but tissues were not thoroughly investigated here; testing of other individuals and tissue types may produce additional positive detections.

To our knowledge, this is the first report of a *Hepatozoon* infection described in giant pandas. The pathogenicity of this novel species and the health and conservation implications for its vertebrate host are unknown. Like many wildlife species found with asymptomatic *Hepatozoon* infections (Clark et al., 1973; McCully et al., 1975; Pawar et al., 2012), the individuals tested here displayed no apparent clinical illness attributable to hepatozoonosis. The high prevalence and penetration into the population demonstrated here may indicate low pathogenicity (Best et al., 2014).

Given the giant panda's vulnerable conservation status, the presence of any identified pathogen warrants consideration of clinical and conservation management implications. Infectious disease outbreaks can have considerable and disproportionate effects on small populations (Castro and Bolker, 2005; Smith et al., 2006). While *Hepatozoon* infection may be an incidental finding in otherwise healthy captive giant pandas, it may have significant

consequences for juvenile, geriatric, or otherwise immunocompromised individuals (Kocan et al., 2000). Concomitant disease or co-infections could precipitate increased parasite load and potentially heightened pathogenicity (McCully et al., 1975; Simposon et al., 2013). Giant pandas are prone to a suite of health issues, including gastrointestinal disorders, infectious diseases, infertility, and others which are incompletely understood (Feng et al., 2016; Qiu and Mainka, 1993; Williams et al., 2016; Zhang et al., 2008). The extent to which a background *Hepatozoon* infection may contribute to illness in these cases is unknown.

Considering a common geographic origin for 13/14 individuals, it is likely that the *Hepatozoon* infections were not locally acquired at receiving institutions but rather were already present in the animals upon arrival. The high prevalence demonstrated here could indicate: an enzootic infection of giant pandas, where they may be the parasite's natural host; a high rate of exposure; or an elevated susceptibility to a novel infection due to either host or agent factors. The genus has a wide distribution globally, including species identified from China (Wei et al., 2016; Xu et al., 2015). Phylogenetic relationships also support a plausible Asiatic origin, as the species clusters with Old World *Hepatozoon* species. Its proximity to *H. ursi* is expected from an evolutionary standpoint and consistent with an Asiatic origin. *Hepatozoon ursi* is a parasite of other Asiatic ursids like Japanese black bears (*Ursus thibetanus japonicas*) and Indian sloth bears (*Melursus ursinus*) (Kubo et al., 2008; Pawar et al., 2011), and to date, North American ursids have not been reported with *Hepatozoon* infections.

The route of transmission remains to be described. Cases of tick-infested free-ranging giant pandas have been reported (Qiu and Mainka, 1993), although possible vector species were not sought or identified in these cases. The positive detection in a neonate suggests a potential vertical transmission route (Allen et al., 2011; Murata et al., 1993). Because host specificity of

this novel *Hepatozoon* species is unknown, the impact of an introduced hemoparasite on local mammalian populations is unclear. The potential risk to both native wildlife and giant pandas may warrant stringent *Hepatozoon* surveillance in the captive population.

Further research is necessary to assess whether this *Hepatozoon* species constitutes a potential pathogen of giant pandas and to characterize the extent of threat to the species. Epidemiologic studies in China may help determine whether there is a correlation between the degree of parasitemia and any occurrence of clinical signs. The parasite's infectivity, pathogenicity, range, prevalence, and vector species in free-ranging populations are unknown, but should remain priorities for future investigation. Caution may also be indicated in the international movement of captive individuals. Additional studies can elucidate pathogen, host, and vector relationships, and identify the need for targeted conservation management actions for this vulnerable species.

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Tables:

Table 1. Summary of *Hepatozoon* PCR results from retrospective and opportunistic testing of captive giant pandas.

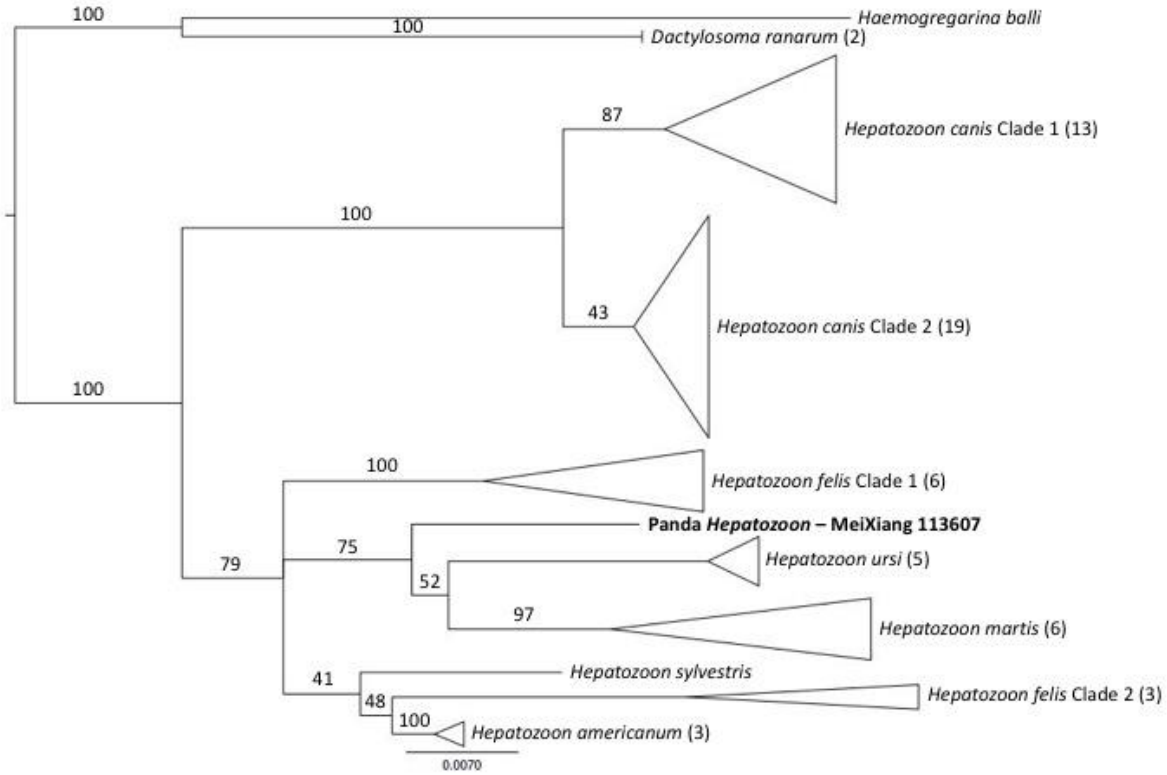
ID	Sex	Status	Origin	Country	Location of Sampling	Country	Sample Type	Collection Date	Positive Detection
GP 1	M	Wild-caught captive	Qionglai Mountains, Baoxing	China	National Zoological Park	USA	Stained blood smear	1983	
							Stained blood smear	1984	
							Plasma	1982	x
GP 2[a]	F	Wild-caught captive	Qionglai Mountains, Baoxing	China	National Zoological Park	USA	Stained blood smear	1985	
							Stained blood smear	1989	
							Kidney	1992	x
GP 3[b]	M	Captive	National Zoological Park	USA	National Zoological Park	USA	Kidney cell culture	1983	x
GP 4[c]	M	Captive	CCRCGP[*]	China	National Zoological Park	USA	Whole blood	2006	
							Stained blood smear		x
GP 5	F	Captive	CCRCGP	China	National Zoological Park	USA	Whole blood		x
							Whole blood	2005	
GP 6	M	Captive	Chongqing Zoological Garden	China	Memphis Zoo	USA	Serum	2006	x
GP 7	F	Captive	Beijing Zoological Garden	China	Memphis Zoo	USA	Whole blood	2006	x
GP 8	F	Captive	Chengdu Research Base	China	Zoo Atlanta	USA	Whole blood	2006	x
GP 9	M	Captive	Chengdu Research Base	China	Zoo Atlanta	USA	Whole blood	2006	x
GP 10	M	Wild-caught captive	Qionglai Mountains, Sanjiang	China	CCRCGP	China	Plasma	1986	x
							WBC	1986	x
GP 11	F	Wild-caught captive	Min Mountains, Nanping	China	CCRCGP	China	Plasma	1986	x
							WBC	1986	x
GP 12	M	Wild-caught captive	Qionglai Mountains, Baoxing	China	CCRCGP	China	Plasma	1986	x
GP 13	F	Wild-caught captive	Qionglai Mountains, Baoxing	China	CCRCGP	China	Plasma	1986	x
							WBC	1986	x
GP 14[d]	M	Wild-caught captive	Qionglai Mountains, Baoxing	China	Zoological Society of London, London Zoo	UK	Plasma	1983	x

[a] Kidney sample acquired post-mortem; individual died due to heart failure.
[b] Denotes neonate; did not survive beyond a few days. Sample obtained post-mortem.
[c] Denotes the individual from which novel *Hepatozoon* species was first detected on light microscopy.
[d] This individual had been temporarily transferred to National Zoological Park, USA in 1981 on breeding loan.
[*] China Conservation and Research Center for the Giant Panda (CCRCGP), Wolong National Nature Reserve

Figures:

Figure 1.

Phylogenetic tree of *Hepatozoon* spp. using *Dactylosoma* and *Haemogregarina* as outgroups, based on available sequences on GenBank. The tree was generated using the GTR + I + G model using Maximum Likelihood. Numbers at the nodes indicate bootstrap values (1000 replicates), and the parasites' vertebrate hosts are labeled along the branches. Based on its phylogenetic position, the giant panda-associated *Hepatozoon* sp. appears most closely related to *H. ursi*, a clade that contains parasites of other Asiatic bears, and *H. martis*. The paraphyletic *H. felis* clades represent parasites of Asiatic lions (*Panthera leo persica*) and leopards (*Panthera pardus*), respectively.



Appendices:

Appendix I. List of all GenBank sequences used in comparative analysis in Fig. 1.

HQ224959.1 *Haemogregarina balli* clone SAS_1 18S ribosomal RNA gene, partial sequence

HQ224958.1 *Dactylosoma ranarum* clone 1B1_6 18S ribosomal RNA gene, partial sequence

HQ224957.1 *Dactylosoma ranarum* clone 1A2_2 18S ribosomal RNA gene, partial sequence

EF622096.1 *Hepatozoon canis* isolate Pelotas 1 18S ribosomal RNA gene, partial sequence

AY461376.2 *Hepatozoon canis* isolate Curupira 1 18S ribosomal RNA gene, partial sequence

DQ439541.1 *Hepatozoon canis* isolate Spain 4 18S ribosomal RNA gene, partial sequence

LC331054.1 *Hepatozoon canis* gene for 18S ribosomal RNA, partial sequence, clone: MoM24

KX712128.1 *Hepatozoon canis* isolate 3470 18S ribosomal RNA gene, partial sequence

KX712124.1 *Hepatozoon canis* isolate 1 18S ribosomal RNA gene, partial sequence

KX712127.1 *Hepatozoon canis* isolate 2734 18S ribosomal RNA gene, partial sequence

KX712125.1 *Hepatozoon canis* isolate 2480 18S ribosomal RNA gene, partial sequence

KX712129.1 *Hepatozoon canis* isolate 3474 18S ribosomal RNA gene, partial sequence

AY150067.2 *Hepatozoon canis* isolate Spain-1 18S ribosomal RNA gene, partial sequence

AY461375.2 *Hepatozoon canis* isolate Curupira 3 18S ribosomal RNA gene, partial sequence

KX712123.1 *Hepatozoon canis* isolate 939 18S ribosomal RNA gene, partial sequence

LC169075.2 *Hepatozoon canis* gene for 18S ribosomal RNA, partial sequence, isolate: MT208

KX712126.1 *Hepatozoon canis* isolate 2733 18S ribosomal RNA gene, partial sequence

DQ439540.1 *Hepatozoon canis* isolate Venezuela 2 18S ribosomal RNA gene, partial sequence

LC331053.1 *Hepatozoon canis* gene for 18S ribosomal RNA, partial sequence, clone: LuM2

AY461378.2 *Hepatozoon canis* isolate Spain 2 18S ribosomal RNA gene, partial sequence

AY731062.1 *Hepatozoon canis* isolate Spain 3 18S ribosomal RNA gene, partial sequence

KC138531.2 *Hepatozoon canis* clone 9617 18S ribosomal RNA gene, partial sequence

KC138532.2 *Hepatozoon canis* clone 9618 18S ribosomal RNA gene, partial sequence

DQ111754.1 *Hepatozoon canis* isolate Dog-26 18S ribosomal RNA gene, partial sequence

LC331052.1 *Hepatozoon canis* gene for 18S ribosomal RNA, partial sequence, clone: ZD7

KU893120.1 *Hepatozoon canis* isolate fox 3-2 18S ribosomal RNA gene, partial sequence

KU893124.1 *Hepatozoon canis* isolate fox 9 18S ribosomal RNA gene, partial sequence

KU893127.1 *Hepatozoon canis* isolate dog 4 18S ribosomal RNA gene, partial sequence

KU893121.1 *Hepatozoon canis* isolate fox 4-2 18S ribosomal RNA gene, partial sequence

KU893126.1 *Hepatozoon canis* isolate dog 3 18S ribosomal RNA gene, partial sequence

KU893123.1 *Hepatozoon canis* isolate fox 6 18S ribosomal RNA gene, partial sequence

KU893119.1 *Hepatozoon canis* isolate fox 2-2 18S ribosomal RNA gene, partial sequence

KU893118.1 *Hepatozoon canis* isolate fox 1-2 18S ribosomal RNA gene, partial sequence

KU893125.1 *Hepatozoon canis* isolate fox 33 18S ribosomal RNA gene, partial sequence

KU893122.1 *Hepatozoon canis* isolate fox 5-2 18S ribosomal RNA gene, partial sequence

HQ829429.1 *Hepatozoon ursi* isolate LaCONES/Indian sloth bear 01 18S ribosomal RNA gene, partial sequence

EU041718.1 *Hepatozoon ursi* isolate Gifu 2 18S ribosomal RNA gene, partial sequence

HQ829434.1 *Hepatozoon ursi* isolate LaCONES/Indian sloth bear 06 18S ribosomal RNA gene, partial sequence

HQ829430.1 *Hepatozoon ursi* isolate LaCONES/Indian sloth bear 02 18S ribosomal RNA gene, partial sequence

EU041717.1 *Hepatozoon ursi* isolate Gifu 1 18S ribosomal RNA gene, partial sequence

AF176836.1 *Hepatozoon americanum* 18S ribosomal RNA gene, partial sequence

AY461377.2 *Hepatozoon sp*. - Curupira 2 18S ribosomal RNA gene, partial sequence

KC127679.1 *Hepatozoon sp*. F3 18S ribosomal RNA gene, partial sequence

LC169077.2 *Hepatozoon sp*. I35 gene for 18S ribosomal RNA, partial sequence, isolate: I35

LC169076.2 *Hepatozoon sp*. MT456 gene for 18S ribosomal RNA, partial sequence, isolate: MT456

EF222257.1 *Hepatozoon sp*. European pine marten 1 18S ribosomal RNA gene, partial sequence

KU198330.1 *Hepatozoon sp*. badger isolate 04/00284 18S ribosomal RNA gene, partial sequence

MG136688.1 *Hepatozoon martis* isolate 446/17 18S ribosomal RNA gene, partial sequence

MG136687.1 *Hepatozoon martis* isolate 197/16 18S ribosomal RNA gene, partial sequence

XXXXXXXXXX *Hepatozoon* (panda) 113607 MeiXiang

KC138534.1 *Hepatozoon felis* clone 1 18S ribosomal RNA gene, partial sequence

KC138533.1 *Hepatozoon felis* clone 8533 18S ribosomal RNA gene, partial sequence

KX017290.1 *Hepatozoon felis* isolate Etawah 18S ribosomal RNA gene, partial sequence

HQ829445.1 *Hepatozoon felis* isolate LaCONES/Bengal tiger 01 18S ribosomal RNA gene, partial sequence

AY628681.1 *Hepatozoon felis* isolate Spain 2 18S ribosomal RNA gene, partial sequence

AY620232.1 *Hepatozoon felis* isolate Spain 1 18S ribosomal RNA gene, partial sequence

KX757032.1 *Hepatozoon silvestris* isolate 152/16 18S ribosomal RNA gene, partial sequence

HQ829439.1 *Hepatozoon felis* isolate LaCONES/Asiatic lion 02 18S ribosomal RNA gene, partial sequence

HQ829444.1 *Hepatozoon felis* isolate LaCONES/Indian leopard 02 18S ribosomal RNA gene,

partial sequence

HQ829438.1 *Hepatozoon felis* isolate LaCONES/Asiatic lion 01 18S ribosomal RNA gene, partial sequence