



SHORT COMMUNICATIONS

First report of *Giardia duodenalis* assemblages A and B in wild hornbills

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ABSTRACT

The Kinabatangan Wildlife Sanctuary is internationally known for its biodiversity and is recognized as one of an Important Bird Area (IBA). There are eight species of hornbills that persist in the fragmented landscape of Kinabatangan, Sabah. While the lack of nesting sites, food resources and hunting threats have been well described, little is known about the health status of these birds in the wild. Accordingly, the present study aimed to determine the occurrence of *Cryptosporidium* and *Giardia* genotypes in the wild populations of hornbills in Sabah, East Malaysia as it may have implications to current conservation strategies. Understanding the distribution of parasites in wildlife is a crucial area of study, as parasites can have a substantial impact on the health and viability of wild animals, as well as zoonotic consequences. At the time of writing, the occurrence of *Cryptosporidium* and *Giardia* protozoa in wildlife has been understudied and poorly documented in Southeast Asia. This study provides the first molecular evidence of zoonotic *Giardia duodenalis* assemblages A and B in hornbills. These findings are important to assess the health of wild populations of endangered hornbills. In addition, to emphasize the importance of monitoring the distribution of assemblages of *G. duodenalis* with a broad host distribution as top priority for the development of appropriate public health policies to reduce the potential transmission of this enteric protozoan.

Keywords: *Cryptosporidium*; *Giardia duodenalis*; Bornean hornbill; Malaysia; hornbill conservation.

INTRODUCTION

There are eight species of hornbills that persist in the fragmented landscape of Kinabatangan, Sabah and they are listed in the Red List as critically endangered Helmeted hornbill (*Rhinoplax vigil*), endangered Wrinkled hornbill (*Rhabdotornis corrugatus*) and White-crowned hornbill (*Berenicornis comatus*), three vulnerable species, i.e., Rhinoceros hornbill (*Buceros rhinoceros*), Black hornbill (*Anthracoceros malayanus*) and Wreathed hornbill (*Rhyticeros undulatus*), near threatened Bushy-crested hornbill (*Anorrhinus galeritus*), and least concern Oriental Pied hornbill (*Anthracoceros albirostris*). From the eight species, the Helmeted hornbill, Wreathed hornbill and White-crowned hornbill have become increasingly rare along the Kinabatangan (Kaur, 2020). Limiting factors for any hornbill population include availability of natural tree cavities (Poonswad, 1993, 1995; Datta & Rawat, 2004), food resources and presence of suitable roosting sites. Logging activities diminish available nesting cavities because hornbills nest in valuable timber trees i.e. Dipterocarp trees (Jinamoy *et al.*, 2014). The scarcity of nesting opportunities for large-bodied hornbills is also made apparent by the continued utilization of artificial nest-boxes in Kinabatangan (Kaur *et al.*, 2020; Vercoe *et al.*, 2021)

While the lack of nesting sites, food resources and hunting threats have been well described, little is known about the health status of these birds in the wild. It is important to investigate the types of parasites these birds may be harboring, and to identify potential threats these endangered birds may be facing in the wild. This is especially important for the Helmeted hornbill, a critically endangered bird with a highly specific diet and nesting requirement (Kaur *et al.*, 2018). The objectives in this study are also aligned with objective 3.1 of the Helmeted hornbill Action Plan (2017) which identifies 'disease screening' as a high priority research and conservation action for the species (Jain *et al.*, 2018).

Cryptosporidium and *Giardia* are enteric protozoa which can be transmitted through ingestion of contaminated food and water sources to humans and animals, including birds. It can affect the upper intestinal tract in humans (Dixon, 2021) causing symptoms such as foul smelling, watery diarrhea. Notably, birds are the known primary host to a few species of *Cryptosporidium* (i.e., *Cryptosporidium ornithophilus*, *Cryptosporidium proventriculi*, *Cryptosporidium galli*, *Cryptosporidium baileyi* and *Cryptosporidium meleagridis*) and several genotypes of *Giardia ardeae* (reviewed in Ryan *et al.*, 2021). In the past decade, there have been numerous reports on the human infective *Cryptosporidium* species in wild

migratory birds (i.e., *Cryptosporidium andersoni*, *C. meleagridis* and *Cryptosporidium parvum*) (Koompaong et al., 2014; Reboredo-Fernandez et al., 2015; Wang et al., 2020; Jian et al., 2021), domesticated poultry (i.e., *C. meleagridis* and *C. parvum*) (Bomfim et al., 2013; Ayinmode & Falohun, 2018; da Cunha et al., 2018; Ferrari et al., 2018) and pet birds (i.e., *C. andersoni*, *C. meleagridis*, *Cryptosporidium muris* and *C. parvum*) (Gomes et al., 2011; Dong et al., 2021; Liao et al., 2021) across the continents of Asia, Africa, Europe and South America. On the other hand, *G. duodenalis* has been reported to occur in wild birds in the past ten years, including the human infective assemblages B, D, and E (Reboredo-Fernandez et al., 2015; Cano et al., 2016; Jian et al., 2021). Additionally, *G. duodenalis* assemblages A and E have also been detected in domesticated and pet birds, respectively (da Cunha et al., 2017; Dong et al., 2021). In a study from Australia, birds such as Budgerigar chicks showed signs of retarded growth, dehydration, and diarrhoea due to *Giardia* infections (Filippich et al., 1998).

To the best of our knowledge, there is a lack of studies detecting both *Cryptosporidium* and *Giardia* infections in wild hornbills. Therefore, to provide insights into the health status of hornbill populations and potential zoonotic implication, the present study aimed to investigate the occurrence of *Cryptosporidium* and *Giardia* genotypes in the wild hornbill populations of Sabah, East Malaysia.

METHODOLOGY

Specimen collection

A total of 13 hornbill fecal samples were collected from nesting pairs of Rhinoceros hornbills, Wrinkled hornbills, Helmeted hornbills, Bushy crested hornbills and Wreathed hornbill nesting sites throughout the Kinabatangan Wildlife Sanctuary (Sabah, Malaysia) in 2017 to 2018 (Figure 1). Each fecal sample was collected from the base of the nests and kept in stool container. They were kept in the fridge at 4°C in the nearest village before transported to the main laboratory at Universiti Malaya in Kuala Lumpur.

PCR amplification and sequence analyses

The fecal samples were then subjected to genomic DNA isolation using the NucleoSpin® Soil kit (Macherey-Nagel, Germany). The extracted DNA was subjected to nested polymerase chain reaction

(PCR) using MyTaq Red Mix (BioLine, Australia), for amplifications of the 18S rRNA gene of *Cryptosporidium* and triosephosphate isomerase (*tpi*) gene of *G. duodenalis*.

For the detection of *Cryptosporidium*, the first PCR utilized the forward primer 18SiCF2 (5'-GAC ATA TCA TTC AAG TTT CTG ACC-3') and the reverse primer 18SiCR2 (5'-CTG AAG GAG TAA GGA ACA ACC-3'). In the second PCR, a fragment of approximately 580 bp was amplified using 1 µl of the first PCR products by using the forward primer 18SiCF1 (5'-CCT ATC AGC TTT AGA CGG TAG G-3') and the reverse primer 18SiCR1 (5'-TCT AAG AAT TTC ACC TCT GAC TG-3'). Both PCR protocols shared the following steps: initial denaturation at 94°C for 3 minutes, followed by forty-five cycles of PCR (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds), and a final extension at 72°C for 10 minutes (Ryan et al., 2003).

For the detection of *Giardia*, the first PCR utilized the forward primer AL3543 (5'-AAATATGCTGCTCGTCG-3') and the reverse primer AL3546 (5'-CAAACCTTITCCGCAAACC-3'). Subsequently, in the second PCR, a 530 bp fragment was amplified using 2 µl of the first PCR products along with the forward primer AL3544 (5'-CCCTTCATCGGIGGTAACCT-3') and the reverse primer AL3545 (5'-GTGGCCACCACICCGTGCC-3'). Both PCR protocols for *Giardia* detection involved an initial denaturation at 94°C for 3 minutes, followed by 35 PCR cycles (94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 60 seconds), and a final extension step at 72°C for 10 minutes (Sulaiman et al., 2003).

The PCR amplified fragments were subsequently sent to a local company (Apical Scientific Sdn Bhd, Kuala Lumpur, Malaysia) for bi-directional sequencing using both forward and reverse primers. Sequences generated from the present study were deposited in the National Center for Biotechnology Information (NCBI) GenBank database under the accession numbers OP729398-OP729399. A maximum likelihood (ML) phylogenetic tree of *Giardia* was plotted using MEGA X (Kumar et al., 2018). The ML bootstrap values were estimated using 1,000 replicates with General Time Reversible + Gamma Distributed model. The nucleotide sequences were aligned with known assemblage reference sequences retrieved from the literature. Additional closest sequences displayed in BLAST and other relevant sequences were also included for phylogenetic tree construction. *Giardia muris* (AF069565) was used as an outgroup.



Figure 1. A male *R. vigil* (left) and a male *B. rhinoceros* (right) perched at their respective natural nest cavities (Photo by Sanjitpaal Singh).

RESULTS AND DISCUSSION

Based on the *tpi* gene of *G. duodenalis*, three out of 13 individual hornbills were amplified and successfully sequenced. Phylogenetic analysis revealed the occurrence of zoonotic assemblage AII (OP729398) in one sample of *R. vigil*, and assemblage BIV in two samples of *B. rhinoceros* (OP729399) (Figure 2). None of the samples were positive for *Cryptosporidium*.

In captivity, hornbills are not usually screened for these protozoans, hence it is not clear if infected birds would display any illness. Little attention has been given to *Cryptosporidium* and *Giardia* infections in birds, especially in Malaysia. To date, there have been only two research studies that reported *Cryptosporidium* infection in captive birds. In 2005, Rohela et al. reported the occurrence of *Cryptosporidium* oocysts through Ziehl-Neelsen staining technique in 28% (28 of 100) of studied captive birds (i.e., Wrinkled Hornbill, Great Argus Pheasant, Black Swan, Swan Goose, Marabou Stork, and Moluccan Cockatoo) at the Malaysian National Zoo. Subsequent confirmation using immunofluorescence technique revealed that 21.4% (6 of 28) were positive to *Cryptosporidium* (Rohela et al., 2005). Two years later, another study was also conducted at the same zoo, in which captive Wreathed hornbill, Great Curassow, Bushy-crested Hornbill and Common Peafowl (4 or 3.4% of 116) were detected positive to *Cryptosporidium* by

Ziehl-Neelsen staining (Lim et al., 2007). Remarkably, detection of oocysts in a bird handler (12.5% of 8) was also reported in this zoo, suggesting that this zoonotic pathogen was acquired during close contact with the infected animals (Lim et al., 2007). However, this postulation remained untested and further molecular tests would be essential to understand how the transmission had occurred.

The present study reported *G. duodenalis* assemblages A and B among studied wild hornbills, though the source of infection is unknown. Nevertheless, these assemblages have been detected in human hosts in Malaysia, including indigenous people (Choy et al., 2014), migrant workers (Sahimin et al., 2018) and HIV patients (i.e., assemblage A) (Lim et al., 2011). In addition, both assemblages have also been reported in domesticated goats (Lim et al., 2013), assemblage B in urban rodents (Tan et al., 2019) and assemblage A in a recreational lake water (Lim et al., 2009) in the country. This study represents the first report of *G. duodenalis* assemblages A and B in hornbills.

The occurrence of *G. duodenalis* in birds has been underreported in Malaysia and in the adjacent countries. However, the human infective *G. duodenalis* assemblages were reported in wild birds in Northern and Northwest Spain, for example, assemblage B in aquatic birds, Common Buzzard, Common Quail and Eurasian Magpie; and assemblage D in Eurasian Jay (Reboredo-Fernandez et al., 2015; Cano et al., 2016). In addition, *G. duodenalis* assemblage A was

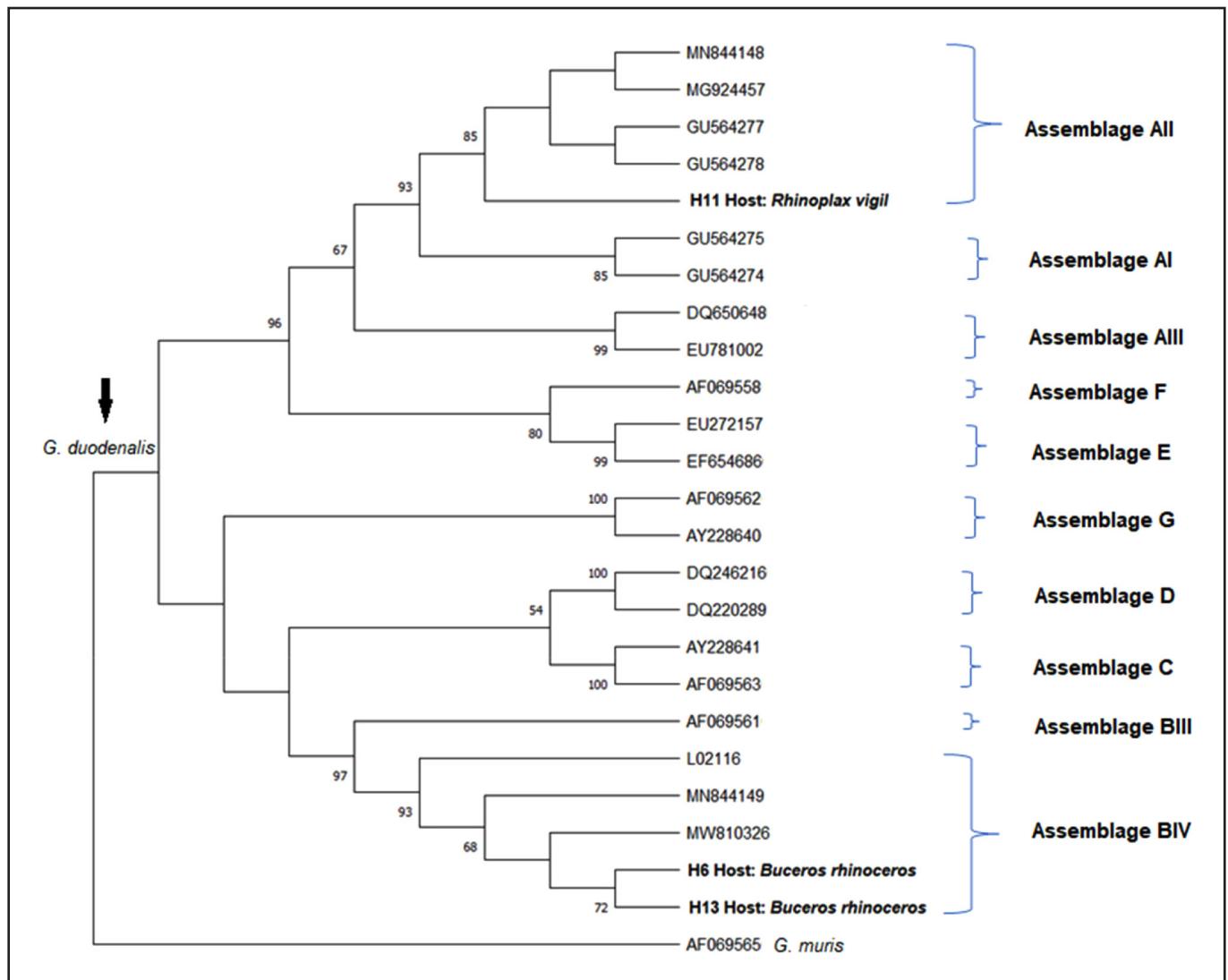


Figure 2. Maximum likelihood phylogenetic tree of *Giardia duodenalis* assemblages based on *tpi* gene sequences. Sequences generated in the present study are in bold.

also reported in captive Toco Toucan from aviary in Brazil (da Cunha et al., 2017). In Asia, *G. duodenalis* ruminant assemblage or E was detected in 33 out of 1005 (3.3%) captive pet birds from trading places in Henan Province, China (Dong et al., 2021). Additionally, low prevalence (3.3%/23 of 679) of assemblages B and E in wild-birds was also reported in Qinghai Lake, Qinghai-Tibetan Plateau (Jian et al., 2021).

In conclusion, this study represents the first molecular evidence of zoonotic *Giardia duodenalis* assemblages A and B in hornbills. Though their potential as a source of infection should not be disregarded, direct contact between humans and wild hornbills are rare, resulting in low risk for zoonosis. In situations where direct contact is anticipated, (i.e. captive zoological setting, confiscation of live birds, etc), transmission can be controlled and minimized through improved hygiene and sanitation (Dixon, 2021). Available evidence suggests that wild birds play a limited role in transmitting infectious diseases in humans (Tsiodras et al., 2008).

Future studies with a larger sample size from wild hornbills are warranted to gain a better understanding of the epidemiology of these parasites in the region, especially when asymptomatic infections are common in both humans and animals (Dixon, 2021). Obtaining this information would enhance the knowledge on potential reservoir for these parasites. This in turn allow the establishment of public health policy to prevent zoonotic transmission while ensuring appropriate conservation strategies are put in place to preserve the biodiversity in the region.

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Conflict of interests

The authors declare no competing interests or financial conflicts of interest.

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