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Lurking in the dark: Cryptic *Strongyloides* in a Bornean slow loris

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ABSTRACT

Within host communities, related species are more likely to share common parasitic agents, and as a result, morphological similarities have led researchers to conclude that parasites infecting closely related hosts within a community represent a single species. However, genetic diversity within parasite genera and host range remain poorly investigated in most systems. *Strongyloides* is a genus of soil-transmitted nematode that has been reported from several primate species in Africa and Asia, and has been estimated to infect hundreds of millions of people worldwide, although no precise estimates are available. Here we describe a case of infection with a cryptic species of *Strongyloides* in a Bornean (Philippine) slow loris (*Nycticebus menagensis*) living within a diverse community of several primate species in the Lower Kinabatangan Wildlife Sanctuary, Malaysian Borneo. Fresh fecal samples were collected from five primate species and nematode larvae cultured from these samples were selected for phylogenetic analyses. Sequences obtained for most larvae were identified as *S. fuelleborni*, grouping into three different clusters and showing no aggregation within specific hosts or geographic location. In contrast, a set of parasite sequences obtained from a slow loris clustered closely with *S. stercoralis* into a different group, being genetically distinct to sequences reported from other primate hosts, humans included. Our results suggest that although *S. fuelleborni* infects all haplorrhines sampled in this primate community, a different species might be infecting the slow loris, the only strepsirrhine in Borneo and one of the least studied primates in the region. Although more data are needed to support this conclusion, we propose that *Strongyloides* species in primates might be more diverse than previously thought, with potential implications for ecological and evolutionary host-parasite associations, as well as epidemiological dynamics.

1. Introduction

We are facing times characterized by unprecedented ecological change. Increasing rates of land conversion and habitat loss coupled with rapid growth of human populations and expansion into new areas pose risks for cross-species transmission of potential pathogens between wildlife populations, livestock and humans (Morgan et al., 2006; Goldberg et al., 2008; Cable et al., 2017; Hassell et al., 2017). Relevant to this context are parasitic nematodes infecting multiple host species, as their partial development outside of the host allows for infective larvae to persist in the environment, facilitating transmission between closely related host species without the need for direct contact or immediate temporal overlap (Morgan et al., 2004; Walker and Morgan, 2014; Nantima et al., 2015). Nematodes, however, tend to be highly overlooked; they are rarely responsible for the mortality of their hosts, causing instead chronic debilitating diseases or even asymptomatic infections that usually perpetuate parasite dispersal and therefore risk of infection. Among such parasites of humans, *Strongyloides* is probably one of the most neglected (Olsen et al., 2009). Due to the challenging aspects of its diagnosis, control measures still have not been included in health packages targeting endemic areas (World Health Organization, 2012), and thus basic aspects of its epidemiology, such as its prevalence and distribution, are most likely highly underestimated.

Two species within the genus *Strongyloides* are known to infect humans and non-human primates (Speare, 1989; Viney and Lok, 2015): *Strongyloides fuelleborni* mainly infects nonhuman primates in Africa and Asia, with occasional human infections being reported (Pampiglione and Ricciardi, 1971; Hira and Patel, 1977; Hasegawa

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et al., 2010), while *S. stercoralis* has a cosmopolitan distribution in tropical and subtropical regions, being endemic in several countries but rarely being described in nonhuman primates outside of captivity (Hasegawa et al., 2010; Labes et al., 2011). Human infections reported from Papua New Guinea have been historically assigned to *S. fuelleborni kellyi* (Kelly et al., 1976), based on morphological similarities, although its taxonomic placement as a subspecies of *S. fuelleborni* is no longer supported by phylogenetic analyses (Dorris et al., 2002). Among other *Strongyloides* species infecting non-human primates, *S. cebus* is considered the only species infecting New World monkeys (Darling, 1911; Little, 1966), and *S. venezuelensis*, which typically infects rodents, has been used to experimentally infect marmosets (de Melo et al., 2012).

The first description of *S. fuelleborni* was done from material collected from chimpanzees and baboons more than 100 years ago (Linstow, 1905). However, the possible co-infection of multiple *Strongyloides* species in the first reports could have misinterpreted the descriptions of the parasite's morphology and life history, leading to the assumption that most cases of *Strongyloides* species infecting non-human primates corresponded to a single species. Nevertheless, the diversity of *Strongyloides* species in non-human primates remains unclear.

One problem in determining a given parasite's host range is that parasites that appear to be shared within host communities may in fact represent multiple cryptic species, each with a restricted host range. Therefore, to understand the complexity of transmission ecology it is not only necessary to consider the broader host community, which will ultimately influence parasite spread through parasite sharing and/or source-sink dynamics, but also the likelihood of cryptic parasite diversity only detectable via molecular analyses.

Borneo is one of the many areas targeted through global gap analyses as being in dire need of further investigation and likely to contain large numbers of undiscovered parasite species (Hopkins and Nunn, 2007; Pedersen and Davies, 2009). At least 10 different primate species are known to live sympatrically in the study area, but little is yet known about their parasite diversity and host range (Salgado Lynn, 2010). Here, we (1) characterize the genetic diversity of *Strongyloides* spp. in five of these primate hosts, including the critically endangered Bornean orangutan (*Pongo pygmaeus*) and endangered proboscis monkey (*Nasalis larvatus*), as well as the silvered leaf monkey (*Trachypithecus cristatus*), the long-tailed macaque (*Macaca fascicularis*), and the vulnerable slow loris (*Nycticebus menagensis*), and (2) report a case of infection with a genetically novel isolate of *Strongyloides* sp. in a free-living slow loris.

2. Materials and methods

2.1. Study area and host species

Sample collection was carried out within Lots 6 and 7 of the Lower Kinabatangan Wildlife Sanctuary (LKWS, 5°10′–5°50′N; 117°40′–118°30′E) in Sabah, Malaysian Borneo (Fig. 1). Backswamp areas consist of low-stature forests and grasslands, while drier areas are characterized by riparian and mixed lowland dipterocarp forests (Azmi, 1998). Flooding occurs seasonally between October and March (Harun et al., 2014). Fresh fecal samples were collected during boat surveys along the Kinabatangan River from proboscis monkeys, silvered leaf monkeys, and long-tailed macaques, while samples from orangutans were collected during tracking and direct observation. In October 2015, a slow loris was captured as part of a radio-collaring project, at which time a fresh fecal sample was collected opportunistically.

2.2. Nematode cultures and collection

Fourteen fresh fecal samples (Nm = 1; Pp = 4; Mf = 3; Nl = 4; Tc = 2), presumably collected from different individual hosts because samples were collected from different geographic areas within Lots 6 and 7 of the LKWS, were selected for coproculture. Larvae were cultured within 12 h of collection at the Danau Girang Field Centre (DGFC) using a Harada-Mori filter-paper method (Harada and Mori, 1995) modified for the field. After 3–4 days, larvae were collected and preserved in 70% ethanol for transportation to the Kyoto University Primate Research Institute. Filariform, infective stage (L_3) larvae of *Strongyloides* spp. were morphologically identified under a stereoscope using standard keys (Anderson, 2000) and individually selected at random for DNA extraction (Nm = 20; Pp = 26; Mf = 33; Nl = 52; Tc = 39).

2.3. DNA extraction and phylogenetic reconstruction

Because feces were collected from the ground below primate groups or sleeping trees and not immediately following observed defecations, and because there are up to 10 primate species living sympatrically in the study area, we conducted host species identification for all fecal samples using molecular tools to avoid misidentification. Total genomic DNA was extracted from each sample using a QIAamp DNA stool mini kit (Qiagen, Japan), and a small fragment of the *cytochrome b* (*cytb*) gene was amplified for all samples, using the primer pair L14724/H15149 and PCR conditions described in Irwin et al. (1991).

DNA was also extracted from individual larvae using a QIAamp DNA micro kit (Qiagen, Japan). PCR was carried out in 15 µl reaction mixtures containing 1.5 µl template, 200 µM of each dNTP, 5 µM of each primer, 0.5 U of Ex-Taq polymerase (Takara) and the manufacturer-supplied reaction buffer. Thermal reactions were performed under an initial denaturation at 96 °C for 2 min, 35 cycles of denaturation at 96 °C for 15 s, annealing at 40 °C for 30 s, and extension at 72 °C for 90 s, followed by a final extension at 72 °C for 7 min. Partial sequences of the mitochondrial *cytochrome c oxidase subunit 1* (*cox1*) gene were amplified using primers StrCoxAfrF (5′-GTGGTTTTGGTAATTGAATGGTT-3′) and MH28R (5′-CTAACTACATAATAAGTATCATG-3′) (Hasegawa et al., 2010; Hu et al., 2003). Products were sequenced in both directions on a 3130 Genetic Analyzer (Applied Biosystems, CA, USA) using the aforementioned primers.

Nucleotide sequences were aligned and adjusted in MEGA 6.06 (Tamura et al., 2013). Published sequences were included in the alignment to identify putative species, and phylogenetic trees were reconstructed using neighbor-joining (NJ) and maximum likelihood (ML) algorithms. Evolutionary distances were computed using the Tamura-Nei model, and bootstrap consensus trees were inferred from 1000 replicates.

2.4. Accession numbers

All sequence data were submitted to the DNA Data Bank of Japan (DDBJ) under accession numbers LC197946-LC198003 and LC317016-LC317043.

3. Results

Species identification was confirmed for all individual hosts. PCR on larval DNA generated amplicons of 716 bp for the *cox1* gene, indicating positive detection of *Strongyloides* spp. for 85 larvae (Nm = 18; Pp = 19; Mf = 18; Nl = 18; Tc = 12). Phylogenetic analyses for both algorithms gave similar results (S1). All parasite sequences from *P. pygmaeus*, *N. larvatus*, *M. fascicularis* and *T. cristatus* were identified as *S. fuelleborni* and grouped together with no correspondence to any particular host species, differing from sequences of *S. fuelleborni* published for other primate species from Japan and several countries in Africa (Fig. 2). Moreover, pairwise distances within the *S. fuelleborni* group indicate a wider cryptic diversity for Bornean isolates than that observed for Japanese and African isolates (Blouin, 2002; Hasegawa et al., 2010). All parasite sequences obtained from *N. menagensis* grouped together into two distinct haplotypes, varying significantly both from sequences isolated from other Bornean primates and from reference