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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

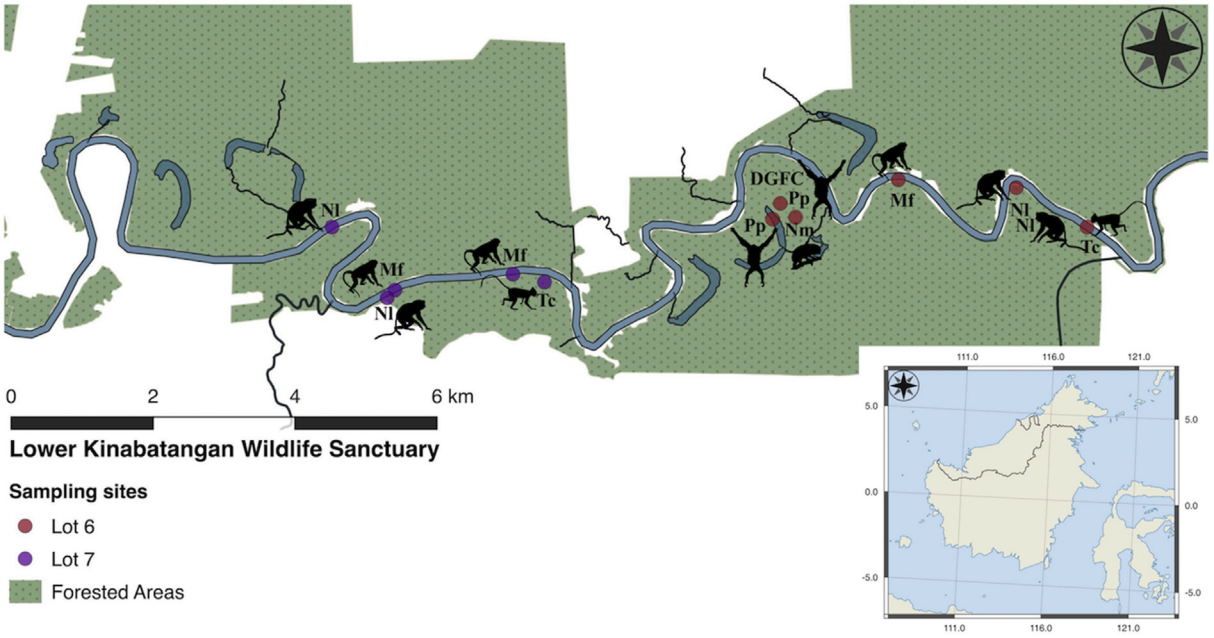

Fig. 1. Sampling sites within Lots 6 and 7 of the Lower Kinabatangan Wildlife Sanctuary, Malaysian Borneo (Nm = *Nycticebus menagensis*, Pp = *Pongo pygmaeus*, Mf = *Macaca fascicularis*, Nl = *Nasalis larvatus, Tc = Trachypithecus cristatus,* DGFC = Danau Girang Field Centre).

sequences identified as *S. stercoralis*. Pairwise distances between all groups, suggest that the slow loris clade may represent a different lineage than that observed to infect free-living non-human primates, humans, domestic dogs and captive chimpanzees (Table 1).

4. Discussion

Parasites circulate in host communities in which species differ in exposure and susceptibility to infection, and thus identifying parasite community composition and transmission networks among species is essential to understand disease transmission and persistence (Fenton et al., 2015). Equally important, however, is to identify cryptic parasite diversity within these communities. Many studies have assumed sharing of parasitic nematodes within primate communities based on morphological descriptions alone, leading to overestimations of cross-infection among closely related species, and by extrapolation, disease risk among them (Gasser, 2006). The incorporation of molecular analyses has highlighted genetic differences among otherwise morphologically indistinguishable nematodes (Poulin, 2011; Ristau et al., 2013; Tan et al., 2012).

In the case of *Strongyloides*, there are still knowledge gaps regarding which species infect primate communities, with most reported cases of infection in nonhuman primates being attributed to *S. fuelleborni* (Chapman et al., 2006, 2007; Gillespie et al., 2005, Gillespie and Chapman, 2006, Gillespie and Chapman, 2008; Hasegawa et al., 2010, 2016; MacIntosh et al., 2010; Rondón et al., 2017; Solórzano-García and de León, 2017). Our study of *Strongyloides* spp. isolates suggests that elements of host generalism and specificity can operate in closely related parasites within the same host community. *S. fuelleborni* was identified in four of the five sympatric primates examined, with the exception of the slow loris. Isolates of *S. fuelleborni* from these Bornean primates showed considerable variation both within and between hosts at both the individual and the species level, clustering into three clades with no specific correspondence to any particular host or geographic location. Pairwise differences in *cox1* among interbreeding nematode strains have been reported to be less than 6%, while differences between distinct species is usually more than 10% (Blouin, 2002). If also true for *Strongyloides* spp., genetic differentiation between clades in the *S. fuelleborni* group would reflect ecological isolation, with shorter distances between Asian clades and larger distances to the African clade. A more comprehensive investigation into *S. fuelleborni* in Bornean primates could help clarify the extent of its diversity in the whole primate community.

Most surprisingly, our phylogenetic analyses also identified a cryptic *Strongyloides* lineage within parasite sequences retrieved from the slow loris. These sequences did not cluster with those examined for other Bornean primates, nor with published sequences of *S. fuelleborni,* and may therefore represent a previously uncharacterized taxon, more closely aligned phylogenetically with *S. stercoralis*. By phylogenetic analysis of *cox1*, isolates of *S. stercoralis* cluster largely by species rather than by geographical regions, except in areas where zoonotic transmission occurs. Thus, alternatively, isolates from the slow loris clade may represent a cryptic subpopulation within a *S. stercoralis* group. High resolution data could help resolve whether these different clades represent different species/subspecies. *Strongyloides* spp. have previously been found in captive slow lorises [*Loris tardigradus*, *N. pygmaeus*, *N. coucang*: Sutherland-Smith and Stalis (2001); *N. pygmaeus*: Streicher (2004)] and in wild Javan slow lorises [*N. javanicus:* Rode-Margono et al. (2015)], but there is no information regarding parasite species diversity within the genus.

Slow lorises are unique in the Kinabatangan primate community as the only representative of the suborder Strepsirrhini, which diverged from the other primate suborder, Haplorrhini, approximately 87 mya (Perelman et al., 2011; Wilkinson et al., 2011). Unfortunately, we have no information concerning the phylogeny of *Strongyloides* spp. in other strepsirrhines, therefore we cannot at present determine whether this cryptic *Strongyloides* sp. is the product of co-speciation with its strepsirrhine hosts. Furthermore, we failed to observe *S. fuelleborni* and the cryptic *Strongyloides* coexisting in the same host, raising the question on how parasite species coexist and assemble into communities, *e.g.* through competitive exclusion, where closely related species are less likely to coexist. Until more samples can be obtained, we cannot conclude the absence of mixed infections in slow lorises, so this is targeted as a key area for future research.

With respect to the remainder of the primate community, further investigation is required to determine the potential of this novel strain to infect other host species, including local humans, and whether populations of slow lorises can enhance parasite persistence through

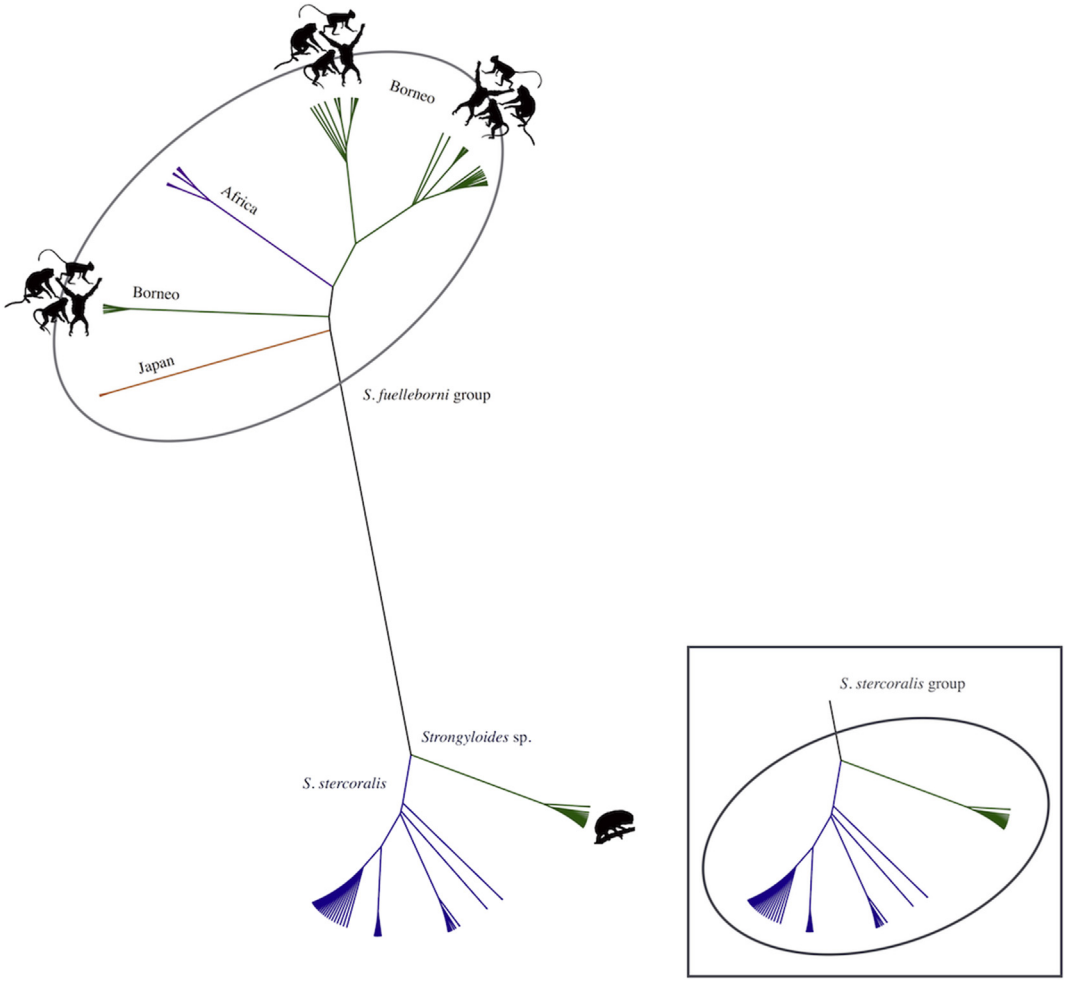

Fig. 2. Simplified neighbor-joining tree reconstructed from partial *cox1* gene (716 bp) sequences of *Strongyloides* spp. *S. fuelleborni* sequences for Bornean primates cluster within the *S. fuelleborni* group, together with previously described sequences for the parasite found in African and Japanese primates. The *S. stercoralis* cluster includes sequences from humans from Laos, Africa and Japan, captive chimpanzees, and dogs. The *Strongyloides* sp. cluster corresponds to sequences from the slow loris. An alternative hypothesis is presented next to the tree, where instead of representing a different species, *Strongyloides* sp. would be part of a cryptic assemblage within the *S. stercoralis* group.

Table 1
Between-group mean distances in *cox1* DNA sequences for 159 isolates of *Strongyloides* spp.

	Parasite species	1	2	3	4	5	6	7
1	*Strongyloides* sp. (slow loris)							
2	*S. fuelleborni* (Borneo, clade 1[a])	0.179						
3	*S. fuelleborni* (Borneo, clade 2)	0.182	0.021					
4	*S. fuelleborni* (Borneo, clade 3)	0.186	0.056	0.061				
5	*S. fuelleborni* (Japan)	0.177	0.061	0.064	0.060			
6	*S. fuelleborni* (Africa)	0.208	0.082	0.084	0.098	0.087		
7	*S. stercoralis*	0.098	0.187	0.187	0.184	0.179	0.202	

[a] Clades were defined based on the neighbor-joining tree presented in S1.

continuous cycles of autoinfection, similar to what happens during infection with *S. stercoralis*. The health consequences of strongyloidiasis might represent an important epidemiological scenario in the years to come, as lorises are popular targets for wildlife trade, increasing people's contact with potential pathogens, and promoting conditions favorable for infectious disease transmission (Chomel et al., 2007; Karesh et al., 2005; Smith et al., 2012). Our results support the existence of generalist *Strongyloides* isolates, but also demonstrate that such generalism does not necessarily extend to the entire host community. Further studies are needed to determine the full extent of the parasite host range and whether there is spatial variation; what determines specificity and generalism in primate-parasite communities, and whether this holds true for other primate communities infected with *Strongyloides* spp. and other intestinal helminth parasites. Furthermore, the development of more robust molecular methods, such as metabarcoding, promises to benefit the assessment of parasite communities in the years to come (Aivelo and Medlar, 2017; Avramenko et al., 2015; Tanaka et al., 2014).

With conflicting evidence concerning the relationship between biodiversity and zoonotic disease (Civitello et al., 2015; Keesing et al., 2010; Levi et al., 2016; Ostfeld and Keesing, 2012; Salkeld et al., 2013; Young et al., 2013), biodiverse and understudied areas represent a priority to understand how infectious diseases can impact wildlife conservation (Daszak et al., 2000; Dobson and Foufopoulos, 2001; Smith et al., 2009). Wild animals, and in particular wild primates, are the source of many organisms that can infect humans (Jones et al., 2008; Wolfe et al., 2007), and even within primates we still know very little about what parasites are in circulation (Cooper and Nunn, 2013; Hopkins and Nunn, 2007). Borneo is a good example of this scenario,

where remarkably little is yet known about parasite diversity and host range in free-living primate communities, despite being targeted as an area with a high likelihood of host shifting among parasites (Pedersen and Davies, 2009). At a time where human translocations and rapid environmental and climate change are dramatically modifying landscapes, we should expect alterations in parasite dynamics and the emergence of new host-parasite associations (Brooks and Hoberg, 2007). Understanding the extent to which parasites are distributed in the wild and the transmission ecology of multi-host parasites is challenging, yet fundamental, to promptly adapt to these changes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2018.03.003.

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