A pinworm's tale: The evolutionary history of *Lemuricola (Protenterobius) nycticebi*

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**ABSTRACT**

*Lemuricola (Protenterobius) nycticebi* is the only pinworm species known to infect strepsirrhine primates outside Africa, and the only pinworm species yet described in slow lorises. Here, we provided a detailed morphological comparison of female and male worms, and a first description of fourth-stage larvae collected from free-living slow lorises (*Nycticebus menagensis*) in Sabah, Malaysian Borneo. Using mitochondrial and nuclear markers, we also reconstructed the species’ phylogenetic relationship with other pinworms infecting primates. Both morphological and molecular results indicated a distinct association between *L.(P.) nycticebi* and its host. However, while taxonomy identified this species as a member of the *Lemuricola* clade and grouped pinworms infecting lemurs and slow lorises together, phylogenetic reconstruction split them, placing *L. (P.) nycticebi* within the *Enterobius* clade. Our results suggest that *L. (P.) nycticebi* may represent a different taxon altogether, and that it is more closely related to pinworm species infecting Old World primates outside Madagascar. *Pongobius pongoi* (Foitová et al., 2008) n. comb. is also proposed.

**1. Introduction**

Pinworms are exceptional among nematodes in that they have conquered both vertebrate and invertebrate realms, having undergone repeated radiations in several hosts (Adamson, 1990). At the same time, their life cycle has remained fairly conservative, characterized by direct transmission and no free-living stages in the external environment. Pinworms' limited dispersal abilities imply that contact with infected conspecifics and reinfection are the most common transmission routes (Cook, 1994; Felt and White, 2005; González-Hernández et al., 2014), virtually “trapping” them in their host lineages over long timescales. This close association between pinworms and their hosts has been extensively studied in primates, where their cophylogenetic structure has led researchers to infer strong patterns of cospeciation, with occasional cross-clade host switching (Cameron, 1929; Sandosham, 1950; Inglis, 1961; Brooks and Glen, 1982; Hugot, 1999; Ashford, 2000, but see Brooks et al., 2015; Nylin et al., 2018).

Oxyurids infecting primates have been classified through standard morphological characters and morphometric variables under the subfamily Enterobiinae, and subdivided into three monophyletic genera, closely underlining the primate classification: *Enterobius* comprising the parasites of the catarrhines, *Trypanoxyuris* comprising the parasites of the platyrrhines, and *Lemuricola* comprising the parasites of strepsirrhines (Hugot, 1999). Only recently was the genus *Pongobius* established based on the description of pinworms parasitizing Sumatran orangutans (Baruš et al., 2007). The genus *Lemuricola* is further divided into three subgenera (Table 1), based on the cephalic and caudal papillae and characteristics of the lips, esophagus and tip of the tail in...
2. Methods

2.1. Study subject and parasite collection

Slow lorises (Nycticebus spp.) are nocturnal arboreal primates distributed throughout Southeast Asia, from northeastern India and southern China to the Thai-Malay Peninsula and extending further south to the islands of Sumatra, Java and Borneo (Baylis, 1928), and later redescribed from a Sunda slow loris (Nycticebus coucang) in Peninsular Malaysia (Inglis and Dunn, 1963).

Here, and almost one hundred years after the original publication, we provide a detailed morphological comparison of L. (P.) nycticebi infecting free-living slow lorises in Sabah (Malaysian Borneo), including a new description of fourth-stage larvae. We then assess its phylogenetic relationship among members of the subfamily Enterobiniinae.

2.2. Morphological observation

The retrieved worms were cleared in glycerol-ethanol solution by evaporating the ethanol from preserved specimens. They were mounted on glass slides with 50% glycerol aqueous solution and observed under an Olympus BX50 microscope equipped with a differential interference contrast apparatus. Free-hand sections were made using a disposable scalpel blade for observation of en-face view of cephalic end and cross slices of the body. Figures were made with the aid of a drawing tube (Olympus U-DA).

2.3. Phylogenetic analyses

Genomic DNA was extracted from two individual female pinworms from two different slow lorises using a QIAamp DNA micro kit (Qiagen, Japan) according to the manufacturer’s instructions. A fragment of the mitochondrial cytochrome c oxidase subunit 1 gene (cox1), the D1 and D2 domains of the 28S ribosomal DNA gene (28S rDNA), and partial 18S ribosomal DNA gene (18S rDNA) were amplified by PCR using the primers shown in Table 2.

Each PCR reaction (15 μl) was prepared using a master mix that consisted of 10 mM buffer, 2.5 mM dNTPs, 5 μM of each primer, TaKaRa Taq HS polymerase (0.5 units), and the DNA template. PCR conditions for the cox1 region consisted of an initial denaturation at 94°C for 2 min, followed by 20 cycles at 94°C for 30 s, 54°C for 30 s, and a final extension at 68°C for 7 min, following Hasegawa et al. (2012); for 28S rDNA gene, an initial denaturation at 94°C for 1 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension for 7 min at 72°C, following Hasegawa et al. (2012); for 18S rDNA gene, an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension for 10 min at 72°C, following Floyd et al. (2005). Following PCR amplification, nonspecific products were removed from the amplicons using the Agencourt AMPure system (Agencourt Bioscience Corp., Beverly, MA), and aliquots were sequenced in an ABI-PRISM 3130 Genetic Analyzer (Perkin Elmer Applied Biosystems, Foster City, CA).

Table 2: Primers used in this study.

| Gene     | Primer name | Sequence (5’-3’)          | Reference          |
|----------|-------------|----------------------------|--------------------|
| cox1     | StrcoxAffF   | GTAATGATGGTGTTTTGGTAATTTGGTT | Hasegawa et al. (2010) |
|          | pr-b        | AGAAGACAGGAGATGAAAATGCAAC   | Nakano et al. (2006) |
| 18S rDNA | Nem18SF     | CGCGAATTCGCCATCACACACGC    | Floyd et al. (2005)  |
|          | Nem18SR     | CCGCGGTTGTCTGCAGCCGCGCC   | de Bellocq et al. (2001) |
| 28S rDNA | C1          | GCCCTGTTTCAAGCCGAGGG       |                    |
|          | D2          | TCCGGGTGTCTGACGGG          |                    |
Genetic Analyzer (Applied Biosystems, CA, USA). Sequences obtained in this study were deposited in the DNA Database of Japan (DDBJ), under accession numbers LC416074-LC416079.

Cox1 and 18S rDNA sequences were aligned using CLUSTALW (Thompson et al., 1994), but multiple sequence alignment for 28S rDNA sequences was conducted in MAFFT to account for the secondary structure of non-coding RNA when constructing the alignment (Katoh and Toh, 2008; Okamoto et al., 2009). Maximum likelihood (ML) and neighbor-joining (NJ) trees were inferred with bootstrap values calculated using 1000 replicates. To provide phylogenetic context to the analysis, we included sequences of cox1, 28S rDNA and 18S rDNA from other members of the Enterobiinae, and also included five sequences of the 28S rDNA gene of L. vauceli and L. bauchoti from feces of ring tailed lemurs (L. catta) collected in Madagascar, and E. vermicularis, T. atelis and T. microon from feces of a captive chimpanzee (Pan troglodytes), black spider monkey (Ateles ater) and night monkey (Aotus azarae), respectively (Accession numbers LC416069-LC416073). *Syphacia frederici* (Oxyuridae: Syphaciaiinae), a parasite of rodents, was used as an outgroup.

3. Results

3.1. Morphological identification

The morphology of adult worms was identical to previous descriptions of *Lemuricola (Protenterobius) nycticebi* by Baylis (1928), Inglis and Dunn (1963) and Hugot et al. (1995), and therefore, only measurements are provided for comparative purposes with those reported by previous studies (Table 3). Because the worm reported as fourth-stage male larva by Inglis and Dunn (1963) was actually in the adult stage, by having fully developed caudal papillae and spicule, fourth-stage larvae are described for the first time as follows.

**Fourth-stage larva**: Body is tapered to both extremities (Figs. 1–3, 8). The cephalic vesicle is absent. Four cephalic papillae, amphibial pores and one pair of minute papillae between amphidial pores are observed; the mouth is triangular and encircled by three lips (Fig. 5). The esophageal isthmus and bulb; e) Ratio to worm length; f) Distance from cephalic end. **Table 3** Morphometric comparison of *L. microon* lemurs (*L.* catta) the 28S rDNA gene of other members of the Enterobiinae, and also included five sequences of the 28S rDNA gene of *L. vauceli* and *L. bauchoti* from feces of ring tailed lemurs (*L. catta*) collected in Madagascar, and *E. vermicularis*, *T. atelis* and *T. microon* from feces of a captive chimpanzee (*Pan troglodytes*), black spider monkey (*Ateles ater*) and night monkey (*Aotus azarae*), respectively (Accession numbers LC416069-LC416073). *Syphacia frederici* (*Oxyuridae: Syphaciaiiinae*), a parasite of rodents, was used as an outgroup.

3.1.1. Taxonomic summary

Host: Philippine slow loris, *Nycticebus menagensis* (Lydekker, 1893).

Locality: Lower Kinabatangan Wildlife Sanctuary (Lot 6), Sabah, Malaysia (5°25′8.00″ N, 118°21′8.43″ E).

Specimens deposited: IBTC PAR-00003 (6 females and 9 males) and PAR-00004 (1 female, and 2 male fourth-stage larvae), Borneensis, Universiti Malaysia Sabah (Kota Kinabalu, Malaysia).

3.2. Phylogenetic analyses

Partial cox1 mtDNA (845 bp), 18S rDNA (761 bp) and 28S rDNA (748 bp) of *L. (P.) nycticebi* were successfully amplified and sequenced. Phylogenetic analyses for each gene consisted of an alignment of 33 sequences trimmed to 636 bp for cox1, 30 sequences trimmed to 740 bp for 18S rDNA, and eight sequences aligned to 828 bp for 28S rDNA, to ensure comparison among homologous regions of the genes. Phylogenetic trees reconstructed by ML and NJ methods yielded similar
topologies, therefore only ML trees are shown (Figs. 9-11). The analysis of both mitochondrial and nuclear genes resulted in trees with similar branching patterns, where *Trypanoxyuris* and *Enterobius* lineages split first. *Cox1* phylogeny further divided *Enterobius* infecting great apes and monkeys, and although not strongly supported, placed *L. (P.) nycticebi* as a different taxon (Fig. 9). The phylogenies for 18S and 28S rDNA gene sequences also confirmed this scenario (Figs. 10 and 11). For the former, there were two *Lemuricola* sequences available other than this study; *Lemuricola* sp., recovered from *Eulemur* sp. in Madagascar, forming a separate cluster than that of *L. (P.) nycticebi*, and *L. pongoi*, infecting Sumatran orangutans, included within the *Enterobius* clade with *E. buckleyi* and *P. hugoti*, which are also orangutan pinworms. Overall, sequences of slow loris pinworms did not form a basal group, branching out from the *Enterobius* lineage instead. This was also the case when other *Lemuricola* species from Madagascar lemurs were included in the analysis (Fig. 11), suggesting that the genus is not monophyletic if *L. (P.) nycticebi* is included.

4. Discussion

The evolution of Oxyurida has been largely driven by the hosts they inhabit and, unlike other nematode lineages, without further diversification in terms of microhabitat or life cycle. The limited dispersal capacity of pinworms further predicts a strong congruence between host and parasite phylogenies, as they have fewer opportunities to encounter new hosts. In the case of primate pinworms, such congruence originates from both parasite- and host-specific attributes that would seem to provide a buffer against host switching and successful exploitation of novel hosts. For instance, even though humans and chimpanzees are closely related, infection with the human pinworm *E. vermicularis* is usually of mild pathogenicity in humans but often results in fatalities in chimpanzees (Murata et al., 2002; Yaguchi et al., 2014). The contrasting infection outcomes in these two closely related species suggests differences in host specificity.

Reports on parasites of free-living slow lorises remain scarce due to the elusive nature of their hosts. However, reports of oxyurids in slow
lorises are not rare and exist for both captive (Sutherland-Smith and Stalis, 2001) and wild individuals (Baylis, 1928; Inglis and Dunn, 1963; Rode-Margono et al., 2015). Unlike studies on pinworms infecting other mammals, sampling pinworms from primates is usually constrained by various factors; e.g. pinworms are uncommonly shed in feces, which can be collected non-invasively, and there are ethical considerations when capture is necessary. By collecting several specimens from different free-living slow lorises in Sabah, we were able to complete previous descriptions of *L. (P.) nycticebi*, including a morphological description of fourth-stage larvae and the genetic characterization of worms.

Classifying biodiversity according to the evolutionary history of different organisms has been a task pursued from the days of the early naturalists, and remains highly relevant and more urgent than ever (Deans et al., 2012) as we keep losing species at unprecedented rates (Dirzo and Raven, 2003; Ceballos et al., 2017). The close association with their primate hosts makes pinworms vulnerable to extinction, particularly when host populations are in decline and density-dependent transmission is compromised (Stork and Lyal, 1993; Dunn et al., 2009; Koh et al., 2004).

Taxonomy, based on the morphological characteristics of organisms, has long been the traditional approach towards classification but now molecular tools are being increasingly used, sometimes leading to discrepancies. Numerous studies of various organisms have documented substantial incongruences between molecular phylogenies and morphological classifications, stimulating controversy over which method should be preferred (Seberg et al., 2003; Tautz et al., 2003; Dunn, 2003; Hebert and Gregory, 2005). In this study, morphological and molecular results agree in that they both indicate a clear association between *L. (P.) nycticebi* and its host. However, differences between the two approaches place *L. (P.) nycticebi* under different phylogenetic scenarios: while taxonomy clusters slow loris and lemur pinworms together, phylogenetic reconstruction of both mitochondrial and nuclear markers places *L. (P.) nycticebi* as a different taxon, distinct from other members of the genus *Lemuricola* and nested within the *Enterobius* clade. Furthermore, the inclusion of *L. pongoi* within the *Enterobius* clade indicates that this species may belong to the *Enterobius* lineage instead of the *Lemuricola* lineage, suggesting greater diversity among orangutan pinworms than previously recognized. It is worth noting that *L. pongoi* possesses a nearly hexagonal cephalic plateau with very large cephalic papillae at four corners in both sexes, an oblong esophageal bulb connecting to the esophageal corpus without strong constriction, and a long tail appendage in the male (Foitzová et al., 2008, 2010). These morphological features suggest close affinity of *L. pongoi* with *Pongobius*, which has been known only from orangutans (see Baruš et al., 2007; Kuze et al., 2010). In phylogenetic analyses, *L. pongoi* also shows a close relationship with *Pongobius* based on sequences of *cox1* and 18S rDNA (Foitzová et al., 2014; this study), and therefore, we would like to propose here a new transfer for this species as *Pongobius pongoi* (Foitzová et al., 2008) n. comb. If the position of *Pongobius* within the lineage of *Enterobius* reflects actual phylogeny, it should be suppressed to subgeneric rank.

Molecular data support the hypothesis that there are three monophyletic clusters within the primate pinworms, however, the *Lemuricola* clade does not seem to be monophyletic. In this regard, not only lorises slow lorises but also their pinworms diverged from their African counterparts ∼40 mya (Perelman et al., 2011; Pozzi et al., 2014), later colonizing Asia. The morphological similarities between pinworms from lemurs and slow lorises may not be the product of a shared
phylogenetic history but instead the result of convergence, *i.e.* independent adaptations to similar environments, in this case their strepsirrhine hosts. If convergence was the actual case, *Protenterobius* should be transferred to *Enterobius* or elevated to generic rank. The subdivisions within the *Enterobius* clade are likely to become clearer with a stronger sampling and sequencing effort. Accurate

A

B

C

Fig. 11. Comparison between (A) primate phylogeny, (B) pinworm phylogeny derived from cladistics, and (C) 28S rDNA gene sequences (outgroup not shown). Female pinworm cephalic ends shown in B correspond to: (top to bottom): *Trypanoxyuris* (Trypanoxyuris) microon (Linstow 1907), *Trypanoxyuris* (Buckleyenterobius) atelis (Cameron 1929), *Enterobius* (*Enterobius*) vermicularis (Linnæus 1758), *Lemuricola* (*Proenterobius*) nycticebi (Baylis 1926), *Lemuricola* (*Madoxyuris*) bauchoti (Chabaud et al. 1965), *Lemuricola* (*Madoxyuris*) vaucelli (Chabaud et al. 1965). Scale: 20 µm. Line drawings reprinted with permission of Cambridge University Press from Hasegawa (2009), Methods of collection and identification of minute nematodes from the feces of primates, with special application to coevolutionary study of pinworms. In: Huffman Chapman (eds.) Primate Parasite Ecology. Cambridge University Press, pp. 29–46.
classifications, involving morphological and phylogenetic descriptions, are the basis for comparative biology, biodiversity studies and conservation efforts, and the identification of phylogenetic associations is part of the exploration and understanding of biological diversity. As they enable the reconstruction of the evolutionary history of organisms, molecular characterization and morphological description should be conducted in concert wherever possible.

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