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First molecular identification of *Strongyloides fuelleborni* in long-tailed macaques in Thailand and Lao People's Democratic Republic reveals considerable genetic diversity

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Research Paper

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Abstract

Strongyloides fuelleborni is a soil-transmitted nematode parasite of non-human primates. The worm is prevalent also in human populations in Africa and South-East Asia. In this study, we amplified and sequenced a portion of the 18S ribosomal RNA gene (rRNA) and of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene of *Strongyloides* adult males recovered from faecal samples from long-tailed macaques (*Macaca fascicularis*) in Thailand and Lao PDR. The prevalence in Thailand was 31.1% (55/177) and in Lao PDR it was 62.1% (41/66), with an overall prevalence of 39.5% (96/243). All 18S rRNA sequences that we obtained (n = 96) showed 100% identity with published *S. fuelleborni* sequences. The 96 *cox1* sequences that we obtained represented 32 new haplotypes. When included with the 17 previously known haplotypes from *S. fuelleborni*, the *cox1* sequences fell into four clusters, which had clear geographical structure. This is the first molecular confirmation of *S. fuelleborni* in long-tailed macaques in Thailand and Lao PDR. Clearly, awareness needs to be raised of the zoonotic potential of *S. fuelleborni*. A monitoring programme should be organized, taking into account the role of reservoir hosts (i.e. monkeys) in the natural background of human strongyloidiasis caused by *S. fuelleborni*.

Introduction

Strongyloides is a genus containing some 50 species of obligate gastrointestinal parasites of vertebrates (mammals, birds, reptiles and amphibians) (Speare, 1989). Two species infect humans: *S. stercoralis* and *S. fuelleborni*-like forms (including *S. fuelleborni kellyi*). The former has a cosmopolitan distribution in tropical and subtropical regions (Schad, 1989) and the latter naturally infects non-human primates in Africa but can also infect humans there (Ashford *et al.*, 1992). Human infection with *S. fuelleborni* was first recorded in Zimbabwe (Grove, 1989), and it subsequently proved to be distributed widely in sub-Saharan countries (e.g. Central African Republic, Cameroon and Ethiopia) (Pampiglione & Ricciardi, 1971; Hasegawa *et al.*, 2010) and South-East Asia, including Thailand (Thanchomnang *et al.*, 2017). This parasite is common among Old-World primates, including rhesus monkeys and macaques (Sandground, 1925). In addition, *S. fuelleborni kellyi* has been reported from humans in Papua New Guinea (Ashford *et al.*, 1992).

The long-tailed macaque (*Macaca fascicularis*) is a species of Old-World monkey that is widely distributed in tropical South-East Asia (Fooden, 1995; Malaivijitnond & Hamada, 2008). Its natural range extends southwards and eastwards from south-eastern Bangladesh and Myanmar, through the southern part of the Indochinese Peninsula (Thailand, Cambodia, Lao PDR and Vietnam) and into Malaysia, Singapore, and the islands of Sumatra, Borneo, Java and the Philippines (Eudey, 2008). In Thailand, the long-tailed macaque is distributed extensively, from the lower northern region to the southern part of the country (Malaivijitnond & Hamada, 2008). Because of human intervention, the habitats of the long-tailed macaque have been greatly altered from natural forests to temple grounds or park areas close to human settlements. The macaque's natural foraging behaviour has also changed, towards begging and searching garbage for food. The possibility of zoonotic transmission of animal parasites from this primate to humans is a real concern. Therefore, we have examined faecal samples of long-tailed macaques inhabiting some tourist attraction sites in Thailand and Lao PDR in a search for

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Fig. 1. Map of the study areas in Thailand and Lao PDR. Numbers and shading indicate study province in each country.

Strongyloides. Recently, *S. fuelleborni* was recorded and identified, based on morphological criteria, in long-tailed macaques from north-east Thailand (Wenz-Mücke *et al*., 2013) and there has been a single report of human infection in the same region (Thanchomnang *et al*., 2017). However, there have been no previous attempts at molecular identification of *S. fuelleborni* in monkeys in Thailand and Lao PDR. Here, we report amplification and sequencing of a portion of the 18S rRNA and *cox1* regions and use the sequences for identification of the *Strongyloides* species present in long-tailed macaques.

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Table 1. *Strongyloides fuelleborni* sequences obtained and analysed in the present study.

Locality (No. of samples)	18S rRNA GenBank accession no.	*Cox1* haplotype	*Cox1* GenBank accession no.	Sample IDs
Maha Sarakham, Thailand (24)	MH045486	H1	MH049697	K5, K43, K92
		H2	MH049698	K14, K24, K40, K48, K54, K71, K108, K110
		H3	MH049699	K23, K33
		H4	MH049700	K36, K47, K50, K75, K86, K106
		H5	MH049701	K53, K77
		H6	MH049702	K58
		H7	MH049703	K94
		H8	MH049704	K95
Udon Thani, Thailand (31)	MH045487	H7	MH049705	S4, S14, S27, S62
		H9	MH049706	S1
		H10	MH049707	S2, S59
		H11	MH049708	S8
		H12	MH049709	S13, S31, S37, S60
		H13	MH049710	S15, S53, S61
		H14	MH049711	S22, S43
		H15	MH049712	S24, S42
		H16	MH049713	S33
		H17	MH049714	S34
		H18	MH049715	S38, S40
		H19	MH049716	S44
		H20	MH049717	S52
		H21	MH049718	S56, S57
		H22	MH049719	S58, S64
		H23	MH049720	S66
		H24	MH049721	S67
Savannakhet, Lao PDR (41)	MH045488	H42	MH049722	L2, L7, L8, L12, L18, L23, L26, L32, L45, L55, L58
		H43	MH049723	L3, L10, L11, L22, L28, L33, L34, L42, L49, L62
		H44	MH049724	L4, L5, L20, L48, L61, L63
		H45	MH049725	L9
		H46	MH049726	L13, L14, L47
		H47	MH049727	L16, L19, L21, L36, L39, L40, L41, L66
		H48	MH049728	L25
		H49	MH049729	L27

Materials and methods

Study areas and populations

Fresh faecal droppings from 243 individual macaques were collected in January and February 2018. Small groups of macaques were followed during their complete daily activity period (06.00–12.00). Individual faecal samples were collected directly after excretion. Faecal samples were collected at Kumphawapi Monkey Park, Kumphawapi District, Udon Thani Province (17°06′41.8″N, 103°01′03.4″E) (n = 67) and Kosumpee Forest Park, Kosumpisai District, Maha Sarakham Province (16°15′190″N, 103°04′024″E) (n = 110) in north-eastern Thailand, and in Champon District, Savannakhet Province, Lao PDR (16°32.608′N, 105°15.081’E) (n = 66). A map showing these localities is provided in fig. 1.

Stool examination and sample collection

Strongyloides free-living adults and larvae were detected using an agar plate culture (Koga *et al.*, 1991). A 3 g portion of each faecal sample (n = 243) was placed on a nutrient agar plate in the field. *Strongyloides* rhabditiform larvae and free-living adults were observed and

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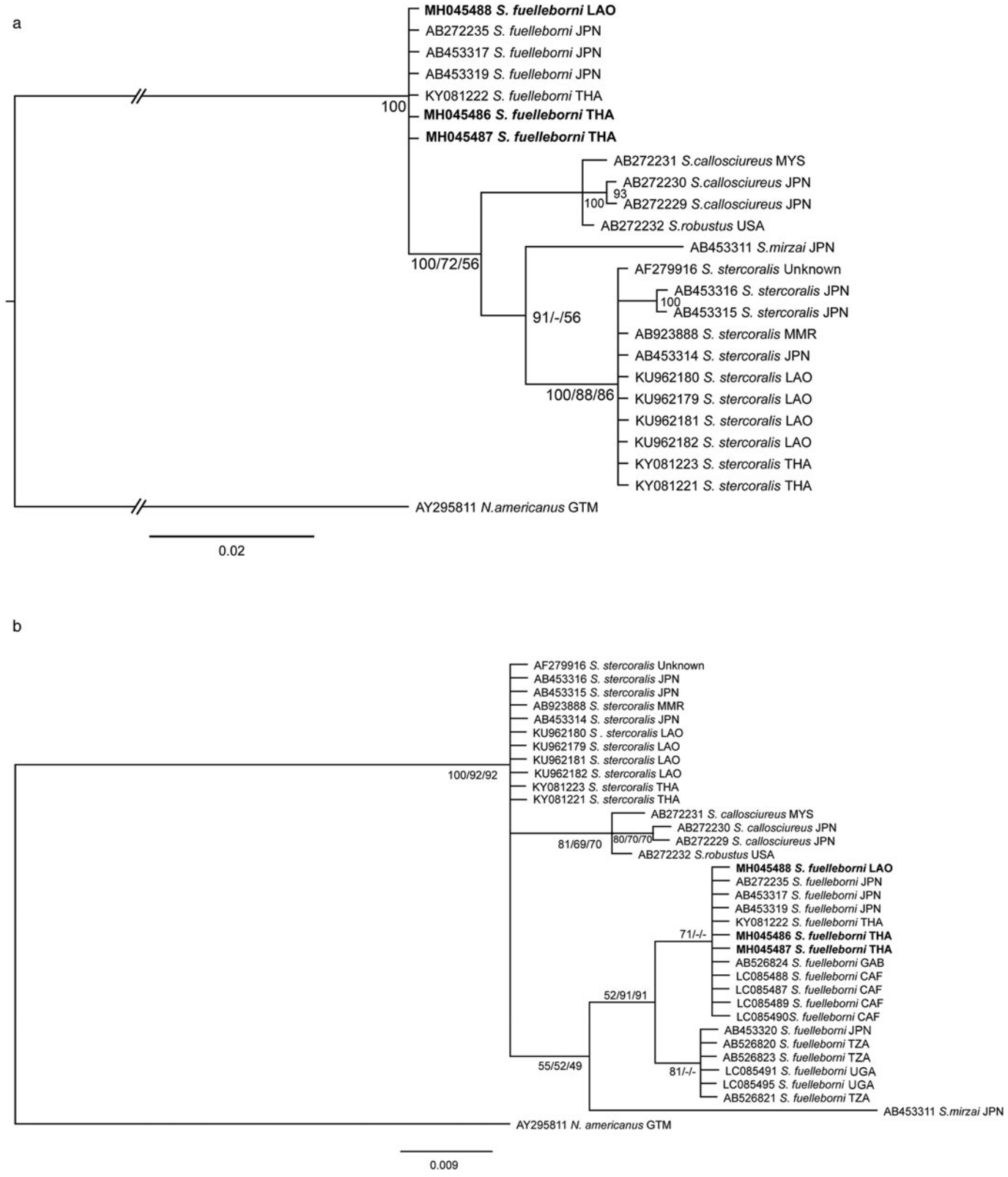

Fig. 2. Maximum-likelihood phylogenetic tree of *Strongyloides* spp. based on partial 18S rRNA sequences. Support values (Bayesian posterior probabilities/ML bootstrap/NJ bootstrap) are shown above the branches. A dash (–) instead of a numerical support value indicates that a particular grouping was not found by that method of analysis. Bold letters indicate sequences obtained in the present study. (a) Phylogenetic tree inferred from a 356-bp alignment of *Strongyloides fuelleborni* from Thailand, Lao PDR and Japan, and (b) a tree inferred from a 219-bp alignment which permitted addition of *S. fuelleborni* sequences from Central African Republic, Gabon, Uganda and Tanzania, which overlap with our sequences by that amount. Sequence AB453320 derived from sample of Japanese researcher, acquired in Tanzania and diagnosed in Japan.

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collected after 3 days of culture at room temperature. Parasite stages harvested were fixed in 70% ethanol, transported to the laboratory, and kept at −20°C until used for molecular identification.

DNA extraction, polymerase chain reaction (PCR), and sequencing

A single free-living adult male worm was taken from each positive culture, individually crushed with a disposable polypropylene pestle (Bellco Glass, Vineland, NJ, USA) and DNA extracted using the Nucleospin Tissue Kit (Macherey-Nagel GmbH & Co, Duren, Germany). Extracted DNA was eluted in 50 µl of elution buffer, 5 µl of which was used for amplification. Primers specific for portions of the *Strongyloides* 18S rRNA gene and of the mitochondrial *cox1* gene were used as previously described (Laymanivong *et al*., 2016). PCR cycling conditions were also as reported in Laymanivong *et al.* (2016). DNA direct sequencing was done using the Applied Biosystems 3730×I DNA Analyzer and ABI Big Dye Version 3.1 (Applied Biosystems, Foster City, CA, USA) in both directions, using the PCR primers as sequencing primers. The gene sequences were analysed using a nucleotide BLAST search via NCBI. New sequences of both regions were aligned with reference sequences from the NCBI. The *cox1* alignment was 646 bp in length. Two different alignments were analysed for the partial 18S gene. The longer one was 356 bp after trimming the primer sequences and reducing the alignment to the length of the shortest published sequence retained in the analysis. A shorter alignment of 219 bp permitted inclusion of additional sequences, primarily from Africa, which overlapped with our sequences by that amount. BioEdit (Hall, 1999) was used for preparing the alignments.

Alignment and phylogenetic analysis

The 18S rRNA sequences of *Strongyloides* spp. were aligned with reference sequences from the NCBI database using BioEdit software (Hall, 1999). Bayesian inference (MrBayes v3.2; Ronquist *et al.*, 2012) was used to construct a phylogenetic tree from the 18S rRNA alignment. Maximum likelihood (ML) and neighbour-joining (NJ) methods, as implemented in MEGA7 (Kumar *et al.*, 2016), were also used. The substitution models for both datasets were chosen using the Bayesian information criterion (BIC) in MEGA7 software: the lowest BIC score is considered to best describe the substitution pattern. For the 18S rRNA alignment, the HKY + G model was selected. MrBayes was run for 500,000 generations, by which time the standard deviation of split frequencies had fallen below 0.01, and trees sampled every 300 generations. In all Bayesian analyses, consensus trees were generated using the command "con type = half compat", which results in a 50 majority-rule tree. For the maximum-likelihood and neighbour-joining methods, the Kimura two-parameter (K2) model was used (Kimura, 1980). Branch support in ML and NJ was assessed by bootstrapping with 1000 replications (Kumar *et al.*, 2016). DnaSP v5 was used to infer haplotype composition and haplotype diversity (Hd) (Librado & Rozas, 2009). A median-joining network was constructed using the Network software version 5.0.0.0 (Fluxus Technology Ltd., www.fluxus-engeneering.com). All sequences have been deposited in the GenBank database (table 1).

Results

In total, 243 faecal samples were collected from free-ranging long-tailed macaques inhabiting three localities in Thailand and Lao PDR. Ninety-six of these (39.5%) were positive for *Strongyloides* by agar plate culture. The prevalence in Thailand was 31.1% (55/177) and in Lao PDR it was 62.1% (41/66). From each positive culture, one adult male free-living worm was used for DNA extraction, amplification and sequencing. The DNA of *Strongyloides* sp. samples was detected with PCR targeting the 18S rRNA and *cox1* genes. Each of the 96 adult male worms possessed an 18S sequence identical to GenBank accessions of *S. fuelleborni* (100% similarity and 100% query coverage; KY081222, AB272235) as reported in studies from Japan and Thailand (fig. 2a). In the tree inferred from the shorter 18S alignment, which permitted addition of partial sequences from Africa, some African sequences were identical to those from Asia, and some were very slightly different, causing them to group in a second clade (fig. 2b).

Similarly, all 96 *cox1* sequences obtained by us showed 98–99% identity with *cox1* sequences of *S. fuelleborni* from humans in Thailand (GenBank accession no. KY081233, 100% query coverage) and 93–94% similarity with sequences of this species from macaques (*Macaca fuscata fuscata*) in Japan (AB526290, AB526291, AB526292, AB526293 and AB526294: 90–91% coverage). Our 96 sequences represented 32 haplotypes, all new. Phylogenetic analysis of *cox1* sequences placed *S. fuelleborni* and *S. stercoralis* in separate clades (100% bootstrap values, data not shown). *Strongyloides fuelleborni* sequences from Thailand and Lao PDR were closely related to those from Japan, but located in separate clades from those from Tanzania, Gabon and Central

Table 2. GenBank accession numbers of previously published *Strongyloides fuelleborni cox1* sequences used to construct the haplotype network.

Haplotype	Cluster	GenBank accession no.	Location
H25	A	KY081233	Thailand
H26	B	AB526294	Japan
H27	B	AB526290	Japan
H28	B	AB526291	Japan
H29	B	AB526292	Japan
H30	B	AB526293	Japan
H31	D	AB526289	Gabon
H32	D	AB526287	Gabon
H33	D	AB526288	Gabon
H34	C	AB526282	Japan
H35	C	AB526284	Tanzania
H36	C	AB526285, AB526286 AB526306	Tanzania
H37	D	LC085508, LC085506	Central African Republic
H38	D	LC085501	Central African Republic
H39	D	LC085502, LC085505	Central African Republic
H40	D	LC085504	Central African Republic
H41	D	LC085503	Central African Republic

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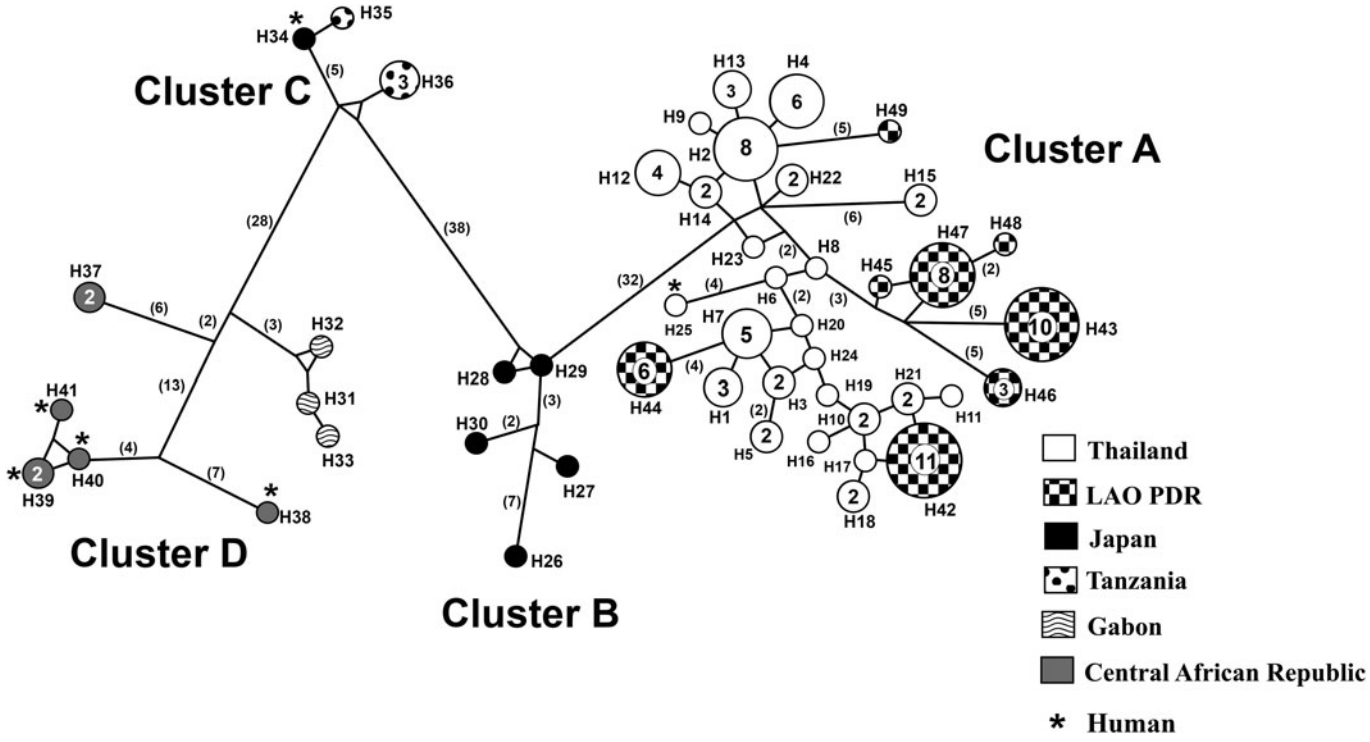

Fig. 3. Median-joining network of *cox1* sequences of *Strongyloides fuelleborni*. The shading patterns indicate the different geographical sources. Numbers in parentheses indicate numbers of nucleotide changes inferred as having occurred between nodes. Numbers in circles indicate number of samples with that haplotype, when this is >1. Cluster A consists of haplotypes 1–24 from macaques (from the present study), haplotype 25 from a human in Thailand (Thanchomnang *et al.* 2017), and haplotypes 42–49 from macaques, collected from Lao PDR (from the present study). Cluster B consists of haplotypes 26–30 from macaques, collected from Japan. Cluster C consists of haplotype 34 from a Japanese researcher who acquired the infection while working with wild chimpanzees in Tanzania, and haplotypes 35–36 from chimpanzees and yellow baboons in Tanzania. Cluster D consists of haplotypes 31–33 from chimpanzees and gorillas, collected from Gabon, haplotype 37 from chimpanzee and gorilla and haplotypes 38–41 from humans, collected from Central African Republic.

African Republic. When other sequences from GenBank were included (table 2), a median-joining network clustered the sequences into four groups (A, B, C and D), with a total of 49 haplotypes (total Hd = 0.9670; Hd from Thailand and Lao PDR = 0.953; Hd from Japan = 1; Hd from Tanzania = 0.7, and Hd from Gabon and Central African Republic = 0.956) (fig. 3). Cluster A consisted of our 32 new haplotypes from Thailand and Lao PDR, together with haplotype 25, a human isolate recovered in Thailand (Thanchomnang *et al.*, 2017). Cluster B consisted of five haplotypes from Japan (macaques). Cluster C consisted of three haplotypes from Tanzania: haplotypes 35 and 36 were from chimpanzees and yellow baboons, respectively, and haplotype 34 was from a Japanese researcher working with wild chimpanzees in the area. Cluster D consisted of three haplotypes from Gabon (chimpanzee and gorilla) and five haplotypes from Central African Republic (humans, chimpanzees and gorillas).

Discussion

Molecular methods for discriminating among *Strongyloides* spp. are important in situations in which the parasite morphology and epidemiological information are similar (Hasegawa *et al.*, 2010, 2016; Thanchomnang *et al.*, 2017). Here, we have presented the first molecular identification of *S. fuelleborni* infections in long-tailed macaques in Thailand and Lao PDR. Sequences from a variable region of the 18S rRNA gene were used to identify *Strongyloides* worms to species. A portion of the *cox1* gene was used to examine genetic diversity within *S. fuelleborni*. Phylogenetic analysis of the 18S rRNA gene can distinguish *S. fuelleborni* from other species of *Strongyloides* (fig. 2). All of the 18S rRNA nucleotide sequences of *S. fuelleborni* from 96 obtained samples were identical with published *S. fuelleborni* sequences from Japan and Thailand. Addition of 18S sequences from Africa in a shorter alignment revealed some African sequences identical with those from Asia and some in a different, but closely related, clade. The 18S gene is known to be highly conserved within species.

However, we found 49 haplotypes among *cox1* sequences from *S. fuelleborni* when sequences from GenBank were included in the analysis. Thirty-three haplotypes were from Thailand and Lao PDR, six from Japan, two from Tanzania, three from Gabon and five from Central African Republic. The haplotypes fell into four separate clusters, each containing examples from humans, except cluster B (macaques in Japan). There was a strong geographical structure among clusters (Hasegawa *et al.*, 2016). Clearly, our sampling efforts in Thailand and Lao PDR have been more extensive than in previous studies in Japan (Hasegawa *et al.*, 2010) and various parts of Africa (Hasegawa *et al.*, 2016). Hasegawa *et al.* (2010) obtained a *Strongyloides* sample from a Japanese mammalogist, who had participated in a field survey in Tanzania. A sequence of the *cox1* gene from this case was almost identical to those of worms in Tanzanian chimpanzees and yellow baboons. The same authors noted that sequences available at the time (n = 24) could be divided into three phylogenetic groups, which corresponded to geographical localities (Japan, Tanzania and Gabon) but not to host species. A later

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analysis by Hasegawa *et al.* (2016) included 14 additional *cox1* sequences of *Strongyloides* larvae obtained from humans, lowland gorillas and chimpanzees inhabiting Central African Republic, and chimpanzees living in Uganda. The haplotypes fell clearly into two lineages, corresponding to *S. stercoralis* and *S. fuelleborni*. *Cox1* haplotypes of *S. fuelleborni* formed clades according to their geographical locality. The *S. fuelleborni* haplotypes from the humans and apes in Central African Republic formed separate clades, closer to those from Ugandan chimpanzees and Gabonese apes, respectively. When aligned with our new sequences, the same pattern was found on a broader scale: geography, rather than host species, dictates the pattern of clustering. The high diversity reported by Hasegawa *et al.* (2016) is matched by the diversity of sequences in our study that the haplotypes of *S. fuelleborni* formed clades according to their geographical locality.

Despite the geographical clustering evident in our *cox1* haplotype network, we cannot infer much about the biogeography of *S. fuelleborni*. The Thai/Lao cluster is closest to the cluster from Japan, but the branch connecting these two clusters is almost as long as the branch connecting the Japanese cluster with African clusters. The long branches separating African sequences suggest that additional sampling there will reveal many additional haplotypes. Primate populations in large parts of Asia also remain to be sampled.

Several molecular methods have been developed for the identification of *Strongyloides* spp. For example, ITS1 and 18S rRNA have been used for PCR-based identification of *S. fuelleborni* and *S. stercoralis* in orangutans and in humans working with wild orangutans in Central and East Kalimantan, Borneo, Indonesia (Labes *et al.*, 2011), 18S rDNA and *cox1* genes were used for identifying *S. fuelleborni* and *S. stercoralis* in humans, several monkey species and dogs (Hasegawa *et al.*, 2010, 2016), 18S rDNA and *cox1* gene sequences for identifying *S. stercoralis* in humans and dogs (Jaleta *et al.*, 2017), and *cox1*, 18S rDNA and 28S rDNA sequences for identifying *S. stercoralis* in humans and dogs (Nagayasu *et al.*, 2017). Recently, *S. fuelleborni* has been reported from humans having contact with long-tailed macaques in Thailand (Thanchomnang *et al.*, 2017). *Strongyloides fuelleborni* has been identified, based on morphological criteria, in long-tailed macaques (*M. fascicularis*) from north-east Thailand (Wenz-Mücke *et al.*, 2013) and confirmed by molecular identification in the present study. These monkeys are now mostly found around temples or recreation parks near human communities (Malaivijitnond & Hamada, 2008).

In conclusion, molecular evidence of *S. fuelleborni* infection in the long-tailed macaque in Thailand and Lao PDR has been presented. The common species infecting humans, *S. stercoralis*, is highly prevalent in human populations in these areas (Laymanivong *et al.*, 2016; Prasongdee *et al.*, 2017). Clearly, awareness needs to be raised of the zoonotic potential of *S. fuelleborni*. Molecular data for systematic, taxonomic and diagnostic studies in human populations at risk of *S. stercoralis* and *S. fuelleborni* infection are important for continuing epidemiological investigations. Such data will also inform prevention and control programmes to reduce animal-to-human transmission in this region. A monitoring programme should be organized, taking into account the role of reservoir hosts (i.e. monkeys) in the natural background of human strongyloidiasis caused by *S. fuelleborni*.

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Conflict of interest. None.

Ethical standards. All procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals and were approved by the Khon Kaen University Ethics Committee for Animal Research (AEMDKKU 001/2018).

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