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Molecular Identification of *Trichuris suis* and *Trichuris trichiura* Eggs in Human Populations from Thailand, Lao PDR, and Myanmar

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Abstract. *Trichuris trichiura* is a soil-transmitted helminth infecting human populations globally. Human cases caused by *Trichuris suis* and *Trichuris vulpis* have also been reported. Molecular identifications of *Trichuris* species infecting human populations in Lao PDR and Myanmar are lacking. Here, we explored molecular data obtained from *Trichuris* eggs recovered from human fecal samples from these countries and compared these with new and existing data from Thailand. Nuclear ribosomal DNA (18S and ITS2) sequences were amplified from *Trichuris* eggs and sequenced. Forty-one samples showed 99–100% similarity in their 18S sequences to published sequences of *T. trichiura* and one sample showed 99% similarity to a sequence of *T. suis*. Similarly, 41 samples showed 92–100% similarity in their ITS2 sequences to published sequences of *T. trichiura* and one sample showed 94–97% similarity to sequences of *T. suis*. This study is the first molecular confirmation of human infection with *T. suis* in northeast Thailand and the first molecular confirmation of the species of *Trichuris* infecting humans in Lao PDR and Myanmar.

INTRODUCTION

Human trichuriasis, caused by *Trichuris trichiura*, is a soil-transmitted helminth infection grouped among important neglected tropical diseases by the World Health Organization.[1,2] An estimated 600–800 million people are infected worldwide.[3] Various *Trichuris* species have been found in mammalian hosts, i.e., *Trichuris suis* (in swine), *Trichuris vulpis* (in canines), *Trichuris ovis* (in sheep), *Trichuris skrjabini* (in goats), and *Trichuris muris* (in mice).[4] Among these, it is thought that only *T. vulpis*[5] and *T. suis*[6,7] can establish persistent active infection in man. Many human cases of trichuriasis present only mild symptoms or are asymptomatic. Persons with heavy infections can experience diffuse colitis, chronic diarrhea, abdominal cramps, rectal tenesmus, and rectal prolapse,[3,8] some of which are important health consequences.[9] Prevalences of human trichuriasis are high in Central Africa, southern India, and Southeast Asia[10] and can be as high as 95% in children,[8] particularly school-age children (5–14 years).[11] In the lower Greater Mekong Subregion, the following prevalences of trichuriasis have been reported: Lao PDR, 8.5%[12]; Myanmar, 57%[13]; Cambodia, 4.1%[14]; and in pregnant women in southern Thailand, 6.3%.[15] Usually, trichuriasis is diagnosed by identification of *Trichuris* eggs in fecal specimens.[16] Eggs of different species of *Trichuris* are very similar, making it difficult to identify species based on the features of eggs.[17]

Molecular techniques are increasingly being used as supportive tools for identification at the species level.[4,7] Several molecular markers are useful for the identification of *Trichuris* spp., such as nucleotide sequences of internal transcribed spacers 1 and 2 (ITS1 and ITS2), nuclear small subunit rRNA (18S rRNA), mitochondrial large ribosomal subunit (*rrnL*), and cytochrome *c* oxidase subunit 1 (*cox1*).[18–20] However, molecular evidence of the species of *Trichuris* infecting human populations in Lao PDR and Myanmar have not been reported yet. Here, we aim to explore such molecular evidence from *Trichuris* eggs recovered from human trichuriasis cases in these countries. We used 18S rRNA sequences to evaluate the phylogenetic relationships of the recovered species from Thailand. The results will provide a better understanding of the molecular epidemiology and a better means for diagnosing human trichuriasis in this region. We placed our data in a global context using sequences of those *Trichuris* spp. publicly available in the GenBank database. We also sequenced the ITS2 region to confirm identities of the recovered *Trichuris* species.

MATERIALS AND METHODS

Study area and sample collection. Forty-two fecal samples were collected from different geographical locations in Thailand (northeast [*N* = 2], north [*N* = 10], and south [*N* = 15]); Lao PDR (Luang Prabang [*N* = 1], Phongsaly [*N* = 1], Champasak [*N* = 2], and Khammouane [*N* = 1]); and Myanmar (Mon State [*N* = 6] and Bago Region [*N* = 4]) (Figure 1). The fecal samples containing *Trichuris* eggs were preserved in 95% alcohol in 15 mL tubes. This study was approved by the Khon Kaen University Ethics Committee for Human Research (HE581292, HE581396, and HE591391). Each participant was informed of the study methods, risks, and benefits of the process. Before enrolment, written consent was obtained from all adult participants and from parents or legal guardians of minors.

Preparation of *Trichuris* species eggs. *Trichuris* eggs were collected using the Mini Parasep method (Apacor, Wokingham, UK). Briefly, 2 grams of positive feces were transferred to a Mini Parasep tube and prepared following the manufacturer's instructions. The *Trichuris* eggs were collected from the fecal sediment in the Mini Parasep tube under a stereomicroscope. Fifty *Trichuris* eggs were collected from

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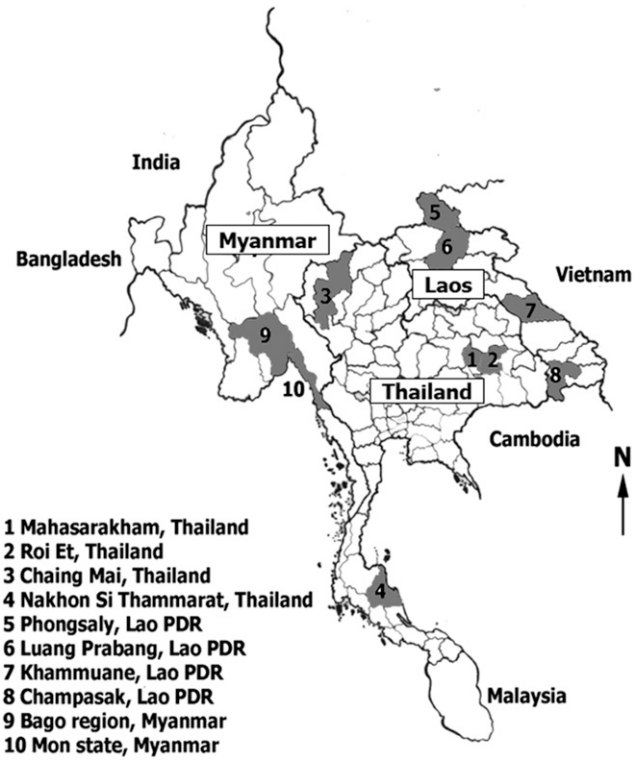

FIGURE 1. Map of the study areas in Myanmar, Lao PDR, and Thailand. Numbers and shading indicate study provinces in each country.

each sample and transferred into a 1.5-mL microcentrifuge tube. All specimens were stored at –20°C until used for DNA extraction.

DNA extraction, amplification, and sequencing. Genomic DNA was extracted from *Trichuris* spp. eggs using the NucleoSpin® tissue kit (Macherey-Nagel GmbH & Co., Duren, Germany), following the manufacturer's instructions. Each PCR assay was performed in a 25-μL reaction volume consisting of 0.625 U of Taq polymerase (Roche Applied Science, Mannheim, Germany), 2.0 mM of $MgCl_2$, 0.2 mM of dNTP mixture (Vivantis, Selangor Darul Ehsan, Malaysia), 0.2 μM of each primer (Sigma-Aldrich, Singapore), and 5 μL of the DNA sample. The PCR conditions and primers are shown in Table 1. The 18S rRNA and ITS2 gene regions were amplified using the GeneAmp PCR System 9700 (Applied Biosystems, Singapore). PCR products were separated in a 1% agarose gel to verify amplification and measure the product length and quantity. DNA sequencing was performed in both directions, using the PCR primers as sequencing primers, with an Applied Biosystems 3730 + l DNA analyzer and an ABI Big Dye version 3.1 cycle sequencing kit (Foster City, CA). All sequences of *Trichuris* spp. were confirmed using a nucleotide BLAST search through the National Center for Biotechnology Information (NCBI).

Phylogenetic tree analysis. The 18S rRNA sequences of *Trichuris* spp. were aligned with reference sequences from the NCBI database using BioEdit software.[21] The maximum likelihood (ML) method of tree construction implemented in MEGA version 6.0 was used.[22] The Tamura 3-parameter (T92 + G) model was found to be the best-fit substitution model. This model was determined using MEGA version 6.0. All sequences were deposited in the GenBank database (GenBank Accession nos. MF288617–MF288648) and are presented in Table 2.

RESULTS

Forty-one of the 42 18S rRNA sequences were very similar (99–100% similarity and 100% query coverage) to various GenBank accessions of *T. trichiura* from humans; AB699090 from *T. trichiura* adult worms from a human in Japan and GQ352547–GQ352555 from eggs in feces of trichuriasis patients in Thailand. The remaining sequence (GenBank Accession no. MF288628), derived from a sample from northeast Thailand, exhibited 99% similarity (100% coverage) to the 18S sequence from a *T. suis* adult from *Sus scrofa domestica* in Spain (GenBank Accession no. HF586905). This sequence showed only 97–98% similarity to *T. trichiura* (coverage 100%; GenBank Accession nos. GQ352547–GQ352555). In the phylogenetic tree (Figure 2), 41 samples were grouped in the clade of *T. trichiura* sequences reported from humans in Japan and Thailand with bootstrap support of 98%. One sample (NE1 THA) (GenBank Accession no. MF288628) was grouped in the clade with *T. suis* from pig in Spain with bootstrap support of 99%. *Trichuris trichiura* and *T. suis* appear as monophyletic sister taxa in the tree (Figure 2).

Similarly, 41 of the 42 ITS2 sequences showed 92–100% identity with ITS2 sequences of *T. trichiura* adults from humans in Uganda (GenBank Accession no. KJ588134, 61–70% query coverage) and China (AM992981 and AM992998, 100%

TABLE 1
The specific primers used in the present study

Gene regions	Primers/approximate amplicon size	PCR conditions
18S rRNA	For Trichuridae nematodes 18S965F: 5′-GGCGATCAGATACCGCCCTAGTT-3′ 18S1573R: 5′-TACAAAGGGCAGGGACGTAGT-3′ (Guardone et al., 2013) PCR product size was 727 bp	1. Pre-incubation 94°C 5 min 2. Amplification for 35 cycles Denaturation 95°C 30 sec Annealing 59°C 30 sec Extension 72°C 30 sec 3. Final extension 72°C 10 min
ITS2	For *T. trichiura* ITS2_tt_F2: 5′-GCTCGTAGGTCGTTGAAG-3′ ITS2_tt_R2: 5′-TAGCCAAGTCGGGTAGT-3′ For *T. suis* ITS2_tt_F2: 5′-GCTCGTAGGTCGTTGAAG-3′ ITS2_tt_R2_new1: 5′-GGGCAGCTTCCGTACT-3′ (newly designed primers) PCR product size of *T. trichiura* was 325 bp and *T. suis* was 355 bp	1. Pre-incubation 94°C 5 min 2. Amplification for 35 cycles Denaturation 95°C 30 sec Annealing 50°C/54°C* 30 sec Extension 72°C 30 sec 3. Final extension 72°C 10 min

ITS2 = internal transcribed spacers 2; PCR = polymerase chain reaction.
* Annealing temperature used was 50°C for *T. trichiura* and 54°C for *T. suis*.

TABLE 2
Trichuris species sequences deposited in the GenBank database

Countries/number of sample	18S rRNA		ITS2	
Thailand (*N* = 27)	Sequence IDs	Accession numbers	Sequence IDs	Accession numbers
Northeastern (*N* = 2)	NE1 THA*	MF288628*	NE1 THA*	MF288646*
	NE3 THA	MF288629	NE3 THA	MF288647
Southern (*N* = 15)	3S THA, 4S THA, 6S THA, 8S THA, 9S THA, 15S THA, 16S THA, 17S THA, 19S THA, 21S THA, 22S THA, 23S THA, 24S THA, 25S THA, and 27S THA	MF288617	4S THA, 6S THA, 8S THA, 9S THA, 15S THA, 16S THA, 17S THA, 19S THA, 21S THA, 22S THA, 23S THA, 24S THA, 25S THA, and 27S THA	MF288633
			3S THA	MF288638
Northern (*N* = 10)	N34 THA, N44 THA, N59 THA, N63 THA, N112 THA, N117 THA, N118 THA, N119 THA and N121 THA	MF288619	N118 THA, N34 THA, N59 THA, N63 THA, N112 THA, N119 THA, N121 THA, and N123 THA	MF288635
	N123 THA	MF288627	N44 THA	MF288644
			N117 THA	MF288645
Lao PDR (*N* = 5)	9PSL LAO	MF288621	9PSL LAO	MF288639
	73CPS LAO	MF288622	73CPS LAO	MF288640
	3LPB LAO	MF288626	3LPB LAO	MF288637
	74KM LAO	MF288623	74KM LAO	MF288641
	81CPS LAO	MF288624	81CPS LAO	MF288642
Myanmar (*N* = 10)	A049 MMR, A037 MMR, A128 MMR, and A149 MMR	MF288618	TS35 MMR, TS45 MMR, TS03 MMR, and TS05 MMR	MF288636
	TS03 MMR and TS35 MMR	MF288620	A037 MMR, A049 MMR, A128 MMR, and A149 MMR	MF288634
	KH086 MMR	MF288625	KH086 MMR	MF288643
	TZY007MMR	MF288632	TZY007MMR	MF288648
	TS45 MMR	MF288631		
	TS05 MMR	MF288630		

ITS2 = internal transcribed spacers 2.
* This sample was identified as *Trichuris suis*, whereas other samples were identified as *Trichuris trichiura*.

coverage). Sample NE1 THA from northeast Thailand—the sample which had an 18S sequence similar to that of *T. suis*—showed 92% similarity to *T. suis* ITS2 sequences from Uganda (GenBank Accession no. JN181780, 62% query coverage) and 94–97% similarity to sequences of this species from China (AM993004, AM993005, AM993011, AM993012, and AM993016, 100% coverage). The same sequence showed at most 85% similarity to *T. trichiura* (GQ301555, 100% query coverage), most of the similarities lying within the 5.8S gene region. The ITS2 regions downstream of the 5.8S gene differed so much between species that alignment was impossible. However, alignment of sequences from individuals within a species was straightforward.

DISCUSSION

Adults of *T. trichiura* and *T. suis* can be discriminated by morphological parameters and biometrical determinations.[23] However, differentiation of the species by egg morphology is difficult. Hence, the development of molecular techniques for species identification and evaluation of diversity is of great use.[19] For example, in 2013, Guardone and coworkers[24] used 18S rRNA and mitochondrial *cox1* gene sequences to discriminate several species of Trichuridae infecting dogs, cats, and wild mammals. The ITS1 and ITS2 and mitochondrial DNA have often been useful for discriminating closely related *Trichuris* species.[25] Here, molecular identification to species of *Trichuris* eggs collected from human fecal samples from Lao PDR, Myanmar, and Thailand has been presented. ITS2 sequences of many *Trichuris* species differ too much for alignment to be possible. For this reason, we did not attempt to construct a phylogenetic tree from these. However, our sequences closely matched those of *T. trichiura* and *T. suis* published previously. Phylogenetic trees of 18S rRNA sequences revealed that 41 and one *Trichuris* samples were closely related to *T. trichiura* and *T. suis,* respectively (Figure 2). Among the species for which sequences are available, these two appeared to be sisters. This result is supported by previous reports from Spain and China that these species are closely related.[7,23] It is, therefore, curious that their ITS2 sequences are so different.

Recently, PCR-restriction fragment length polymorphisms have been used as a tool for the detection of soil-transmitted helminthes including *T. trichiura* in Vietnam, a country located in the lower Greater Mekong Subregion.[26] Adult *T. trichiura* from human and *T. suis* from swine in China were identified by their ITS1 and ITS2 sequences.[7] In Thailand, Areekul and coworkers[27] reported molecular evidence of *T. trichiura* and *T. vulpis* eggs in human fecal samples using PCR. Here, we have reported the first molecular identification of *T. trichiura* in humans in Lao PDR and Myanmar and of *T. suis* from a human in Thailand. A possible human infection with *T. suis* has been reported from the Massachusetts General Hospital, USA: identification of the worm was by morphological features.[6] Successful experimental cross infections of humans with *T. suis* eggs and of pigs with *T. trichiura* eggs have been reported.[28]

Although *T. vulpis* or *T. suis* can cause an uncommon zoonosis and are rarely found in human populations,[5–7] in this study, human species *T. trichiura* and *T. suis* infections were confirmed in humans by molecular identification, but no *T. vulpis* infection was detected despite when considering living environments of human populations in the study areas and sanitary condition, domestic dogs and pigs are probably kept and local people seem to be frequently infected with *T. vulpis* than *T. suis* infection. This reason cannot be

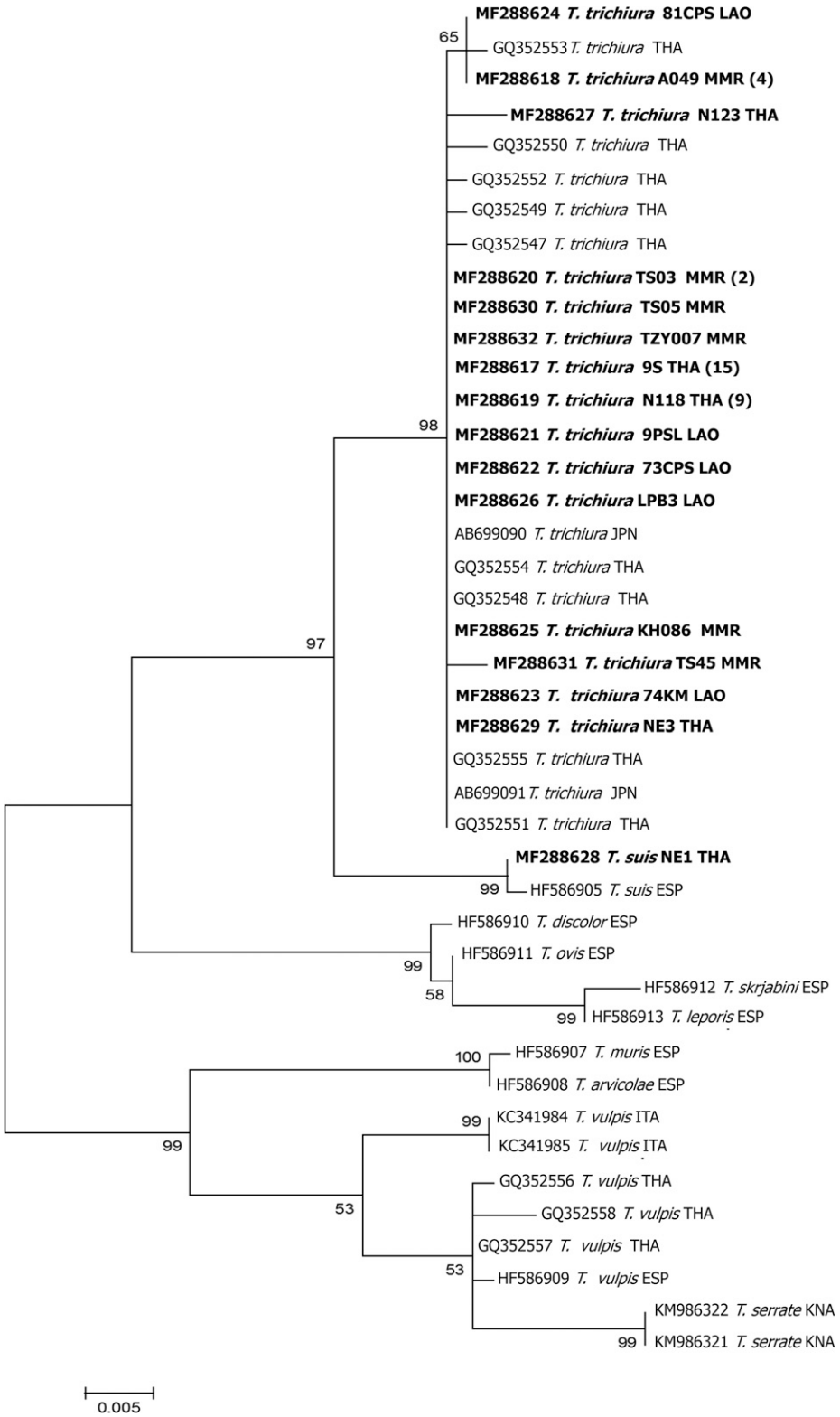

FIGURE 2. Maximum-likelihood reconstruction of phylogeny based on nucleotides (after trimming the primer sequences and reducing the alignment to the length of the shortest published sequence retained in the analysis.) in the 18S rRNA gene of *Trichuris suis* and *T. trichiura*. Bootstrap scores (percentages of 1,000 replications) are presented for each node. The sequences of *Trichuris* species obtained from the GenBank database are indicated with their accession numbers, genus and species, and country codes. *Trichuris* sequences of this study are presented in bold. The tree is midpoint-rooted. CHN = China; ITA = Italy; JPN = Japan; KNA = Saint Kitts and Nevis; LAO = Lao PDR; MMR = Myanmar; THA = Thailand.

accurately explained. This is possibly because of the limited number ($N = 42$) of samples examined in this study. If high numbers of *Trichuris* egg samples are examined, confirmation of *T. vulpis* infection in humans in these areas can possibly be achieved and further observations explored.

In conclusion, molecular evidence of *T. trichiura* and *T. suis* in humans in Lao PDR, Myanmar, and Thailand have been presented. Clearly, awareness needs to be raised of the zoonotic potential of *T. suis* in Thailand. Molecular data, for systematic, taxonomic, and diagnostic studies in human populations associated with *T. trichiura* and *T. suis*, are important for continuing epidemiological investigations, population genetics of *T. trichiura* and *T. suis*, and prevention and control programs to reduce animal-to-human transmission in this region. A monitoring scheme should be established, taking into account the role of reservoir hosts (i.e., pig) in the natural background of human trichuriais caused by *T. suis*.[28]

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