




# Molecular identification of *Ascaris lumbricoides* and *Ascaris suum* recovered from humans and pigs in Thailand, Lao PDR, and Myanmar

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## Abstract

*Ascaris lumbricoides* is the largest roundworm known from the human intestine while *Ascaris suum* is an internal parasite of pigs. Ascariasis, caused by *Ascaris lumbricoides*, has a worldwide distribution. Here, we have provided the first molecular identification of *Ascaris* eggs and adults recovered from humans and pigs in Thailand, Lao PDR, and Myanmar. We amplified and sequenced nuclear ribosomal DNA (ITS1 and ITS2 regions) and mitochondrial DNA (*cox1* gene). Sequence chromatograms of PCR-amplified ITS1 region revealed a probable hybrid genotype from two human ascariasis cases from Chiang Mai Province, northern Thailand. All complete ITS2 sequences were identical and did not differ between the species. Phylogenetic trees and haplotype analysis of *cox1* sequences showed three clusters with 99 haplotypes. Forty-seven samples from the present study represented 14 haplotypes, including 7 new haplotypes. To our knowledge, this is the first molecular confirmation of *Ascaris* species in Thailand, Lao PDR, and Myanmar. Zoonotic cross-transmission of *Ascaris* roundworm between pigs and humans probably occurs in these countries.

**Keywords** *Ascaris* species · Pig · Human · Mitochondrial DNA · Nuclear ribosomal DNA · Molecular identification

## Introduction

*Ascaris lumbricoides* is the largest nematode (roundworm) parasitizing the human intestine and it is one of the principal soil-transmitted helminthic human infections worldwide, infecting an estimated 819 million people (Pullan et al. 2014; Holland 2013). Ascariasis has been considered as a neglected tropical

disease by WHO and is highly prevalent in poor urban and rural areas (Dold and Holland 2010). *Ascaris suum* is an intestinal roundworm of pigs but can cause human ascariasis (Arizono et al. 2010). The two species are similar morphologically and closely related. Indeed, there is debate as to whether they are distinct species (Leles et al. 2012; Shao et al. 2014). *Ascaris*-infected pigs have been reported as a reservoir host for humans

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in both endemic and non-endemic areas such as in China (Zhou et al. 2012; Cavallero et al. 2013; Dunn et al. 2016). *Ascaris lumbricoides* (identified using mitochondrial DNA sequences) has been found in chimpanzees and gibbons in a zoological garden in southwest China (Xie et al. 2013). There have also been reports of the pig *Ascaris* from chimpanzees (Nejsum et al. 2006, 2010). However, cross-transmission of *A. lumbricoides* and *A. suum* between humans and pigs is still debated (Nejsum et al. 2010, 2017a). A pig farmer in northwest Italy was found to be infected with hybrid *Ascaris suum/lumbricoides*. This was determined using PCR-RFLP analysis of the ribosomal nuclear ITS regions (Dutto and Petrosillo 2013). Hybrid genotypes were also found in 32% (30/93) of *Ascaris* adult worms obtained from pigs in the USA (Jesudoss Chelladurai et al. 2017). Experimental cross-transmission of *Ascaris* species between humans and pigs is known (Betson et al. 2014). This implies that control of these parasites requires attention to pig infections as well as human infections.

Several molecular epidemiological investigations, using polymorphic markers (internal transcribed spacer 1; ITS1, mitochondrial cytochrome C oxidase subunit 1; *cox1*, NADH dehydrogenase subunit 1; *nad1* and microsatellite markers), have explored the specificity of the two *Ascaris* species for their respective hosts and their taxonomic status (Cavallero et al. 2013; Iñiguez et al. 2012; Leles et al. 2009). Furthermore, knowledge on transmission dynamics and hybridization of *Ascaris* species has been gained by DNA sequencing, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), and microsatellite marker techniques (Arizono et al. 2010; Cavallero et al. 2013; Jesudoss Chelladurai et al. 2017; Betson et al. 2014).

In Thailand, Lao PDR, and Myanmar, adjacent countries in Southeast Asia, this soil-transmitted helminth is common and poses a public-health problem (Dunn et al. 2016; Laymanivong et al. 2014; Jex et al. 2011; Hlaing et al. 1984). However, molecular confirmation of the identities of *Ascaris* spp. in these areas is still lacking. To fill this gap in our knowledge, we sequenced partial ITS1, complete ITS2 (these are the internal transcribed spacers of the nuclear ribosomal gene repeat), and partial mitochondrial *cox1* sequences from *Ascaris* samples from these countries and prepared a phylogenetic tree and haplotype network of *cox1* sequences. Comparisons with sequences in public databases provide a better understanding of the genetic diversity and identities of *Ascaris* populations in Thailand, Lao PDR, and Myanmar.

## Materials and methods

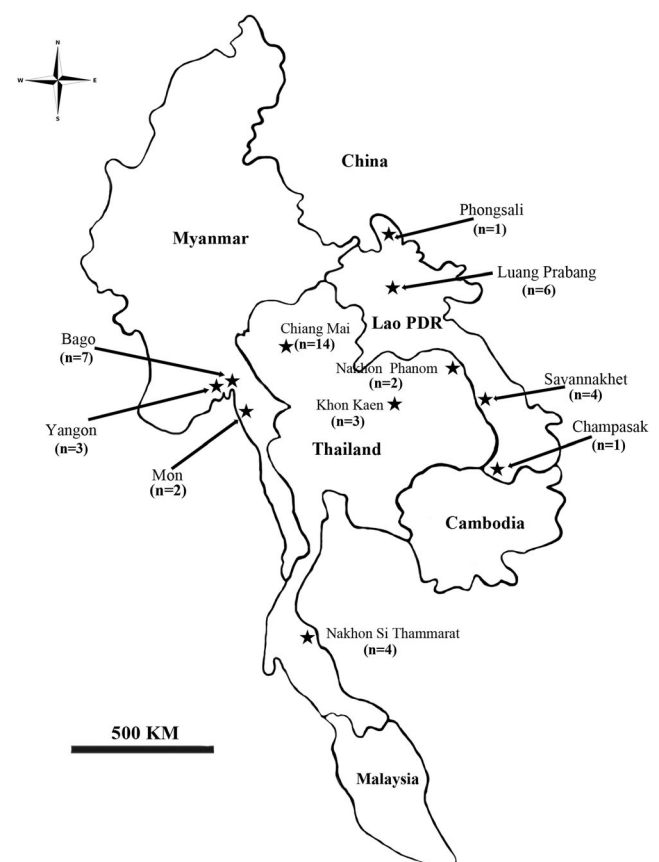
### *Ascaris* samples and ethics approvals

The study used mainly pre-existing material recovered from diagnostic specimens acquired during routine laboratory investigations, and from fecal specimens from patients who

had visited the hospital for treatment: documented informed consent was not required by the ethics committee. Thirty-eight human fecal samples containing *Ascaris* eggs were received from Thailand ( $n = 18$ ), Lao PDR ( $n = 8$ ), and Myanmar ( $n = 12$ ). An additional five (human = 2, pigs = 3) and four (pigs = 4) *Ascaris* adult worms were collected from Thailand and Lao PDR, respectively (Fig. 1 and Table 1). Samples were kept in 70% alcohol and stored at  $-20\text{ }^{\circ}\text{C}$  until used. This study was approved by the Human Ethics Committee of Khon Kaen University (Reference no. HE591403). Patient anonymity was maintained. Specimens of *Ascaris* adult worms from pigs obtained from local slaughterhouses do not correspond to the criteria for observational and field studies, and therefore, a separate ethics clearance was not required for these. No vertebrate specimens were used.

### DNA extraction

Genomic DNA was extracted from a portion of tissue from each adult worm. The tissue was homogenized and then processed using a NucleoSpin® tissue kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's



**Fig. 1** Map showing collection sites of *Ascaris* samples from Thailand, Lao PDR, and Myanmar. This map was modified from a map in The World Factbook, published by the Central Intelligence Agency (Central Intelligence Agency [US] (2017)

**Table 1** *Ascaris* samples from Thailand, Lao PDR, and Myanmar

Sample codes	Number of samples	Stages	Hosts	Regions
H1Ne-THA <sup>a</sup> , H2Ne-THA	2	Adult	Human	Nakhon Phanom, northeastern Thailand
P1Ne-THA <sup>b</sup> , P2Ne-THA,	2	Adult	Pig	Khon Kaen, northeastern Thailand
P3N-THA	1	Adult	Pig	Chiang Mai, northern Thailand
P1C-LAO <sup>c</sup> , P2C-LAO,	4	Adult	Pig	Savannakhet, central Lao PDR
P3C-LAO, P4C-LAO				
H18N-THA <sup>d</sup> , H20N-THA,	14	Egg	Human	Chiang Mai, northern Thailand
H29N-THA, H37N-THA,				
H50N-THA, H51N-THA,				
H52N-THA, H53N-THA,				
H56N-THA, H58N-THA,				
H61N-THA, H62N-THA,				
H68N-THA, H70N-THA				
H10S-THA <sup>e</sup> , H11S-THA,	4	Egg	Human	Nakhon Si Thammarat, southern Thailand
H12S-THA, H13S-THA				
H4N-LAO <sup>f</sup> , H10N-LAO,	6	Egg	Human	Luang Prabang, northern Lao PDR
H16N-LAO, H20N-LAO,				
H21N-LAO, H22N-LAO				
H9N-LAO	1	Egg	Human	Phongsali, northern Lao PDR
H73S-LAO <sup>g</sup>	1	Egg	Human	Champasak, southern Lao PDR
H081B-MMR <sup>h</sup> ,	7	Egg	Human	Bago Region, Myanmar
H084B-MMR,				
H114B-MMR,				
H128B-MMR,				
H184B-MMR,				
H1166B-MMR,				
H1182B-MMR				
H067M-MMR <sup>i</sup>	2	Egg	Human	Mon State, Myanmar
H007M-MMR				
H101Y-MMR <sup>j</sup>	3	Egg	Human	Yangon, Myanmar
H027Y-MMR,				
H023Y-MMR				
Total	47			

<sup>a</sup> H 1Ne-THA Human no. 1 from northeastern, Thailand

<sup>b</sup> P 1Ne-THA Pig no. 1 from northeastern, Thailand

<sup>c</sup> P 1C-LAO Pig no. 1 from central, Lao PDR

<sup>d</sup> H 18N-THA Human no. 18 from northern, Thailand

<sup>e</sup> H 10S-THA Human no. 10 from southern, Thailand

<sup>f</sup> H 4N-LAO Human no. 4 from northern, Lao PDR

<sup>g</sup> H 73S-LAO Human no. 73 from southern, Lao PDR

<sup>h</sup> H 081B-MMR Human no. 081 from Bago region, Myanmar

<sup>i</sup> H 067M-MMR Human no. 067 from Mon State, Myanmar

<sup>j</sup> H 101Y-MMR Human no. 101 from Yangon, Myanmar

protocol. *Ascaris* eggs were collected from fecal sediment after processing with a Mini Parasep® SF fecal parasite concentrator (Apacor, Wokingham, England). *Ascaris* eggs ( $n = 30$  per sample) were homogenized and DNA extracted using a QIAamp® DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA was eluted in 50  $\mu$ l of elution buffer and stored at  $-20^{\circ}\text{C}$  until used.

### Polymerase chain reaction amplification and DNA sequencing

Three specific primer sets were used to amplify ITS1 (Ishiwata et al. 2004), ITS2 (newly designed), and *cox1* (Peng et al. 2005) regions (Table 2). Optimum conditions for amplification with each set are summarized in Table 2. The

**Table 2** The specific primers and PCR conditions for amplification of ITS1, ITS2, and *cox1* of *Ascaris* samples from Thailand, Lao PDR, and Myanmar

Gene regions	Primers and approximate amplicon size	PCR conditions
ITS1	F2662 5'-GGCAAAAGTCGTAACAAGGT-3' R3214 5'-CTGCAATTCGCACTATTTATCG-3' (Ishiwata et al. 2004) PCR product size was 580 bp	1. Pre-incubation 95 °C 5 min 2. Amplification for 35 cycles - Denaturation 95 °C 30 s - Annealing 55 °C 30 s - Extension 72 °C 30 s 3. Final extension 72 °C 10 min 4. Cooling 4 °C ∞
ITS2	AsI2F 5'-TAGCGGTGGATCACTCGG-3' AsI2R 5'-AAGGATTCAGCGTTGGGC-3' (In this study) PCR product size was 550 bp	1. Pre-incubation 95 °C 5 min 2. Amplification for 35 cycles - Denaturation 95 °C 30 s - Annealing 60 °C 30 s - Extension 72 °C 30 s 3. Final extension 72 °C 10 min 4. Cooling 4 °C ∞
<i>cox1</i>	As-Co1F 5'-TTTTTGGTCATCCTGAGGTTTAT-3' As-Co1R 5'-ACATAATGAAAATGACTAACAAC-3' (Peng et al. 2005) PCR product size was 431 bp	1. Pre-incubation 95 °C 5 min 2. Amplification for 35 cycles - Denaturation 95 °C 30 s - Annealing 49 °C 30 s - Extension 72 °C 30 s 3. Final extension 72 °C 10 min 4. Cooling 4 °C ∞

PCR was done using a Gene Amp® PCR System 9700 (Applied Biosystems (ABI), Singapore). Each reaction contained 2.5 µl of 10× high fidelity PCR buffer, 2.0 µl of MgCl<sub>2</sub> (25 mM), 0.5 µl of each deoxyribonucleotide triphosphate (dNTP) mixed (10 mM), 0.5 µl of each primer (10 µM) (Sigma-Aldrich, St. Louis, MO), 0.125 units of *Taq* high-fidelity PCR system (Roche Applied Science, Mannheim, Germany), and 2 µl of DNA template. Deionized water was added to a final volume of 25 µl. PCR products (2 µl) were separated by electrophoresis using 1% agarose gels, stained with ethidium bromide, and the fragment sizes determined by comparison with a 100 bp DNA ladder (Promega™). DNA sequencing was done by First BASE Laboratories Sdn Bhd (Selangor, Malaysia) using the BigDye terminator v3.1 cycle-sequencing kit (ABI). PCR primers were used as sequencing primers and PCR products were sequenced in both directions.

### Sequence alignment, network, and phylogenetic tree analyses

*Ascaris* nucleotide sequences were aligned using the multiple sequence alignment program ClustalW (Thompson et al. 1994) available within BioEdit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) (Hall 1999). Sequence comparisons of the ITS1 region were used for differentiation of *A. lumbricoides* and *A. suum* (Zhu et al. 1999). The complete ITS2 nucleotide sequences (272 bp) were compared with

those of other *Ascaris* spp. from the GenBank database. The *cox1* sequence dataset included 47 sequences generated in this study and 156 from the GenBank database. Phylogenetic tree reconstruction of the *cox1* alignment was done using Bayesian inference in the program MrBayes v3.2 (Ronquist et al. 2012). The substitution models for *cox1* dataset were chosen using the Bayesian Information Criterion (BIC) in MEGA7 software: the lowest BIC score is considered to best describe the substitution pattern (Kumar et al. 2016). For the *cox1* sequence alignment, the Hasegawa-Kishino-Yano (HKY) model with non-uniformity of evolutionary rates among sites (+G) was selected. Analysis of the *cox1* alignment was run for three million generations (2 runs, each of four chains), by which time the standard deviation of split frequencies had fallen below 0.01. Trees were sampled every 1000 generations. Maximum-likelihood (ML) and neighbor-joining (NJ) methods were implemented in MEGA7 (Kumar et al. 2016). The HKY + G model was used for the ML method, while the Tamura-Nei (TN93 + G) model was used for the NJ method (Tamura and Nei 1993). Branch support was provided for ML and NJ trees by bootstrapping with 1000 replications (Kumar et al. 2016). *Anisakis simplex* (JN786328) and *Toxocara vitulorum* (FJ664617) from GenBank database were included in the analysis to serve as out-groups. A median-joining network was based on *cox1* gene sequences of

*Ascaris* using an alignment trimmed to the shortest sequence (327 bp) available in the public database (Supplementary Table S1). Haplotypes were identified in DnaSP version 5.10 (Librado and Rozas 2009). DnaSP was used to prepare an input file for Network software version 5.0.0.1 (Fluxus Technology Ltd., [www.fluxus-engineering.com](http://www.fluxus-engineering.com)) (Bandelt et al. 1999). The latter was used to generate a median-joining haplotype network.

## Results

### ITS1 sequence analysis

Partial sequences of the ribosomal ITS1 region (~452 bp) were used to distinguish between *A. lumbricoides* and *A. suum* in Thailand, Lao PDR, and Myanmar. The two species differ at six positions in this spacer region (Table 3). Differences at

**Table 3** *Ascaris* genotypes, as determined by sequence comparisons of the ITS1 region

Samples	GenBank Accession nos.	Host	Nucleotides position (452 bp alignment)						Species identification
			20	21	22	129	133	248	
H 1Ne-THA	MF358963	Human	–	–	–	–	G <sup>a</sup>	T <sup>b</sup>	<i>A. lumbricoides</i>
H 2Ne-THA	–	–	–	–	–	–	G	T	–
P 1Ne-THA	MF358937	Pig	A <sup>c</sup>	A	A	T	C <sup>d</sup>	A	<i>A. suum</i>
P 2Ne-THA	MF358944	–	A	A	–	T	C	A	–
P 3N-THA	MF358947	–	A	A	A	T	C	A	–
P 1C-LAO	–	–	A	A	–	T	C	A	–
P 2C-LAO	–	–	A	A	–	T	C	A	–
P 3C-LAO	MF358950	–	A	A	A	T	C	A	–
P 4C-LAO	MF358943	–	A	A	–	T	C	A	–
H 18N-THA	–	Human	–	–	–	–	G	T	<i>A. lumbricoides</i>
H 20N-THA	MF358960	–	–	–	–	–	S <sup>e</sup>	W <sup>f</sup>	Probable hybrid AI/As
H 29N-THA	MF358949	–	–	–	–	–	G	T	<i>A. lumbricoides</i>
H 37N-THA	–	–	–	–	–	–	G	T	–
H 50N-THA	–	–	–	–	–	–	S	W	Probable Hybrid AI/As
H 51N-THA	–	–	–	–	–	–	G	T	–
H 52N-THA	–	–	–	–	–	–	G	T	–
H 53N-THA	–	–	–	–	–	–	G	T	–
H 56N-THA	–	–	–	–	–	–	G	T	–
H 58N-THA	–	–	–	–	–	–	G	T	–
H 61N-THA	–	–	–	–	–	–	G	T	–
H 62N-THA	–	–	–	–	–	–	G	T	<i>A. lumbricoides</i>
H 68N-THA	–	–	–	–	–	–	G	T	–
H 70N-THA	–	–	–	–	–	–	G	T	–
H 10S-THA	–	–	–	–	–	–	G	T	–
H 11S-THA	–	–	–	–	–	–	G	T	–
H 12S-THA	–	–	–	–	–	–	G	T	–
H 13S-THA	MF358941	–	–	–	–	–	G	T	–
H 10N-LAO	–	–	–	–	–	–	G	T	–
H 14N-LAO	–	–	–	–	–	–	G	T	–
H 16N-LAO	–	–	–	–	–	–	G	T	–
H 20N-LAO	–	–	–	–	–	–	G	T	–
H 21N-LAO	–	–	–	–	–	–	G	T	–
H 22N-LAO	MF358959	–	–	–	–	–	G	T	–
H 9N-LAO	MF358945	–	–	–	–	–	G	T	–
H 73S-LAO	MF358951	–	–	–	–	–	G	T	–
H 081B-MMR	–	–	–	–	–	–	G	T	–
H 084B-MMR	–	–	–	–	–	–	G	T	–
H 114B-MMR	MF358948	–	–	–	–	–	G	T	–
H 128B-MMR	–	–	–	–	–	–	G	T	–
H 184B-MMR	MF358938	–	–	–	T	–	G	T	–
H 1166B-MMR	–	–	–	–	–	–	G	T	–
H 1182B-MMR	–	–	–	–	–	–	G	T	–
H 067M-MMR	MF358940	–	–	–	–	–	G	T	–
H 007M-MMR	–	–	–	–	–	–	G	T	–
H 101Y-MMR	–	–	–	–	–	–	G	T	–
H 027Y-MMR	–	–	–	–	–	–	G	T	–
H 023Y-MMR	MF358952	–	–	–	–	–	G	T	–

<sup>a</sup> G represent guanine

<sup>b</sup> T represent thymine

<sup>c</sup> A represent adenosine

<sup>d</sup> C represent cytosine

<sup>e</sup> S represent G/C polymorphism

<sup>f</sup> W represent A/T polymorphism

positions 133 and 248 were of most use to distinguish between the species (Fig. 2 and Table 3). All samples from pigs were identified as *A. suum* on this basis. Similarly, almost all human samples were *A. lumbricoides*. However, two human-derived *Ascaris* samples from Chiang Mai Province, Thailand, showed double peaks in chromatograms at positions 133 (Fig. 2a and Table 3) and 248 (Fig. 2b and Table 3), indicating probable hybrids between *A. suum* and *A. lumbricoides*. Representative ITS1 sequences ( $n = 16$ ) have been submitted to the GenBank database (Table 3).

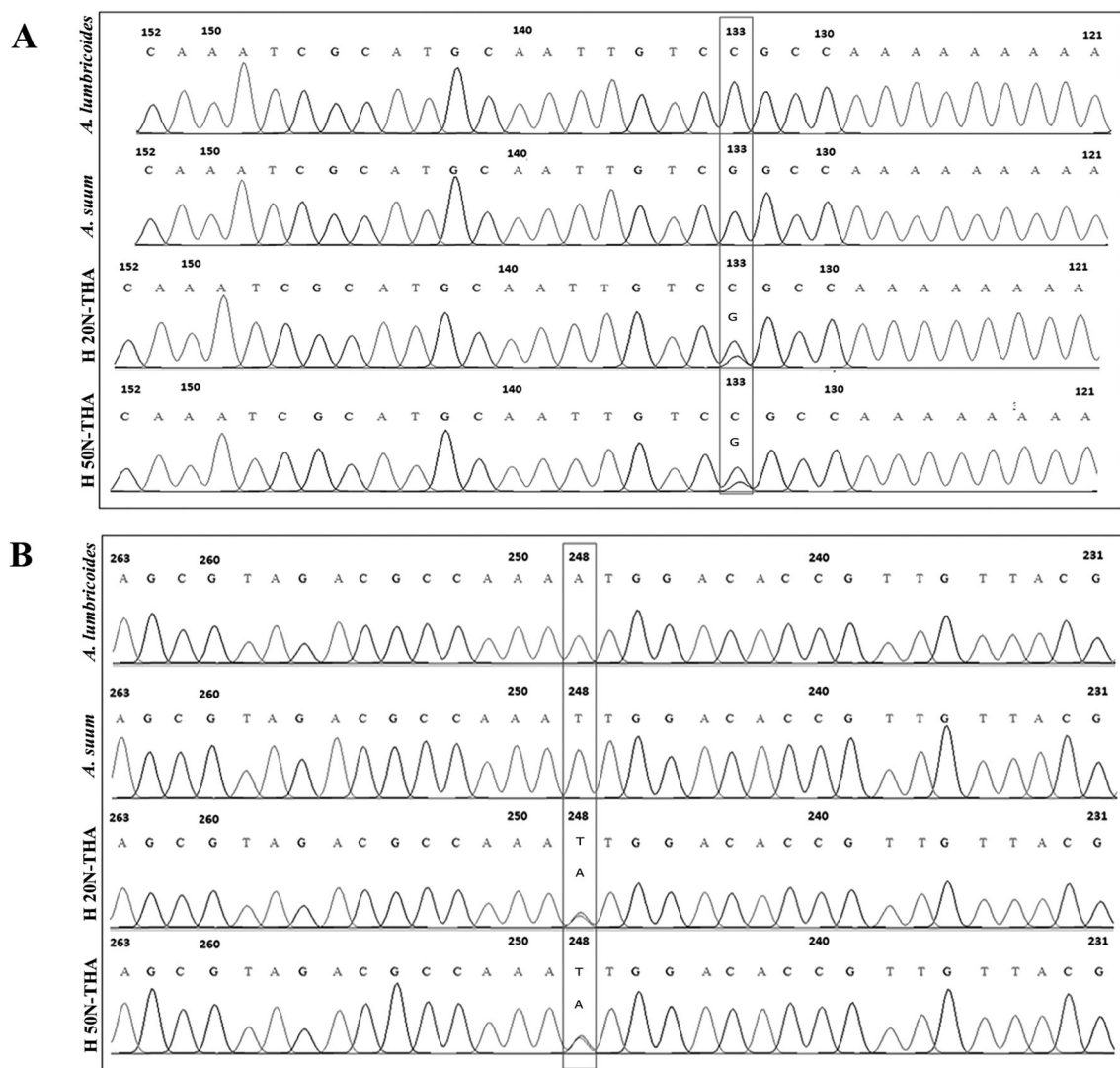
### Analyses of ITS2 regions

Our newly designed ITS2 primer pair (Table 2) amplified a 550 bp product encompassing the 5.8 s, ITS2 and 28 s rDNA gene regions. The complete ITS2 sequences are 272 bp in

length. This region is not informative for differentiation between the two species. Representative ITS2 sequences have been submitted to the GenBank database (GenBank accession nos. MF358936, MF358939, MF358942, MF358946, MF358953–MF358958, MF358961, and MF358962).

### Phylogenetic tree and network analysis of the *cox1* region

A 431 bp PCR product was obtained from all 47 samples sequenced as part of this study. The alignment used was trimmed to 327 bp (the length of the shortest sequence) and contained our new sequences and 156 representative sequences from GenBank (Supplementary Table S1). Representative *cox1* sequences ( $n = 32$ ) from Thailand, Lao PDR, and Myanmar have been submitted to the GenBank



**Fig. 2** Sequence chromatograms of PCR-amplified ITS1 region from two cases (H 20N-THA (accession no. MF358960) and H 50N-THA) of ascariasis from the northern part of Thailand. Probable hybrid genotypes of *Ascaris* species are indicated by double peaks (boxed),

with C or G and A or T at positions 133 (a) and 248 (b), respectively. H, human; N, northern, THA, Thailand; H 20N-THA, collected from human No. 20 from northern Thailand and H 50N-THA, collected from human No. 50 from northern Thailand

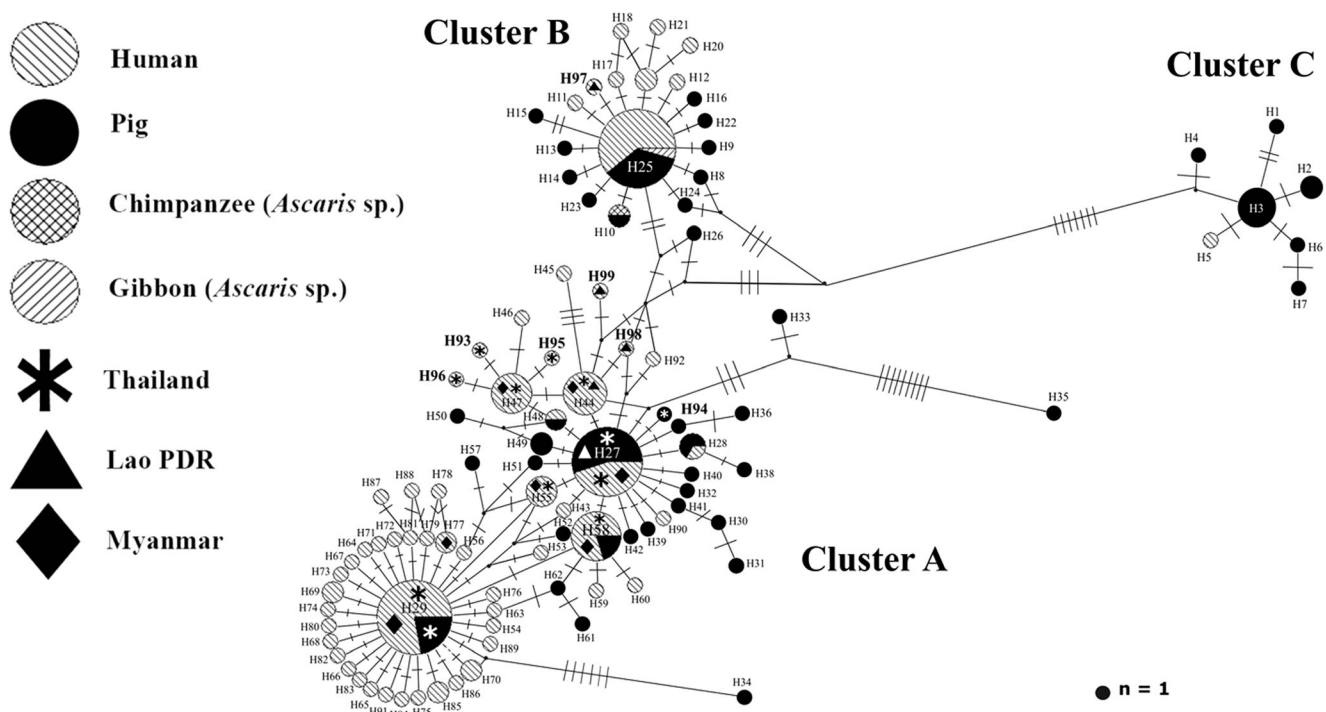
database (GenBank accession nos. MF358904 - MF358935). The median-joining network (Fig. 3) shows three clusters (A, B, and C) corresponding very closely to those in the phylogenetic tree (figure not shown). Ninety-nine haplotypes were recognized in this analysis (haplotype diversity 0.9599) (Fig. 3 and Supplementary Table S1). More *Ascaris* samples fell into cluster A than into clusters B and C. Our new sequences represented 14 haplotypes, seven of which have been previously reported (H27, H29, H44, H47, H55, H58, and H77), and seven of which are new (H93–99). Our new haplotype 97, from Luang Prabang, Lao PDR, was grouped in cluster B, a cluster in which *Ascaris* spp. from natural hosts (chimpanzee and gibbon) are also placed. The remaining six new haplotypes were in cluster A.

The most common haplotype among our sequences was H27 (25.53%; 12/47). Six of our H27 sequences came from Thailand (and included one sample of hybrid genotype), four from Lao PDR, and two from Myanmar. Seven samples were from humans and five from pigs, consistent with previous reports from the USA, Japan, United Kingdom, Philippines, and Uganda (Supplementary Table S1). *Ascaris* eggs from humans recovered from Myanmar ( $n = 5$ ) and Thailand ( $n = 1$ ) were classified in H29, along with published human sequences ( $n = 12$ ) from Brazil, Angola, Tanzania, Zambia, Kenya, Bangladesh, and Uganda and pig sequences ( $n = 5$ ) from USA, Brazil, Uganda, China, and Thailand (from the present study). Haplotype H44 consisted

only of sequences from *Ascaris* recovered from humans ( $n = 8$ ) from China ( $n = 1$ ), Thailand ( $n = 1$ ), Lao PDR ( $n = 5$ ), and Myanmar ( $n = 1$ ).

## Discussion

*Ascaris lumbricoides* and *A. suum* are among the most common soil-transmitted helminths and cause serious health and socioeconomic problems in humans and loss of production in pigs. Evidence of cross-transmission of these species between humans and pigs has been reported (Cavallero et al. 2013), and there is no doubt that the two species share a recent common ancestor (Betson et al. 2013). The host-preference of the common ancestor and the timing and geographical location(s) of the split have been much debated, but the evolutionary histories of human and pig *Ascaris* are likely to be complex and interwoven (Betson et al. 2013). Molecular epidemiology must be used to distinguish and classify these parasites and to clarify the extent of zoonotic transmission. Despite the importance of ascariasis in Thailand, Lao PDR, and Myanmar, there has been no previous work on molecular species identification. Here, we examined the identities of *Ascaris* samples collected from humans and pigs based on sequences of three gene regions. Partial ITS1 nucleotide sequences have two positions that can be used to distinguish between *A.*



**Fig. 3** Median-joining network of *cox1* sequences of *Ascaris* spp. *Ascaris cox1* sequences from this study ( $n = 47$ ) and GenBank sequences ( $n = 156$ ) were distributed in three clusters (A, B, and C). Nucleotide differences are indicated by hatch marks across the connecting lines

(each hatch mark representing a single nucleotide difference). The seven new haplotypes (H93–H99) are shown in black bold text. Accession numbers of sequences from the present study are shown in Supplementary Table S1

*lumbricoides* and *A. suum* (Fig. 2 and Table 3). Two *Ascaris* egg samples from humans in northern Thailand yielded ITS1 sequences suggesting that they were from probable hybrid worms. Previous workers have mentioned the same two variable positions and have detected likely hybrids (Leles et al. 2009; Zhu et al. 1999; Peng et al. 2003). Among our samples, 38 from humans corresponded to the G1 genotype (human-associated genotype), two from humans corresponded to the G5 genotype (probable hybrid genotype), and all 7 samples from pigs represented the G3 genotype (pig-associated genotype, Peng et al. 2003). Additional differences between *A. suum* and *A. lumbricoides* in the ITS1 region include a tract near the 5' end in which there is a run of up to 13 consecutive "A"s. In human-associated *Ascaris*, we found the number of "A"s to always be 10, as also noted by Zhu et al. (1999). In pig-associated worms, the number varies from 11 to 13 but Zhu et al. (1999) showed 12 consecutive "A"s. We prefer to place no importance on this region in attempting to discriminate between *A. suum* and *A. lumbricoides*. Two of these "A"s correspond to positions 20 and 21 in Table 2 of Peng et al. (2003). The question arises as to whether hybrids between the two *Ascaris* species are fertile. If they are as fertile and fecund as the two parent species, gene flow between the species might simply cause them to merge into a single gene pool. S e et al. (2016) have argued that the gene pools remain largely distinct and that hybrids likely have low, or no, fertility (S e et al. 2016). In such cases, hybrids would not transmit genes to future generations and would be of little epidemiological significance. On the other hand, occasional fertile hybrids might lead to introgression between the gene pools, with possible outcomes as discussed by Charles and Criscione (2013).

ITS1 sequence data from *Ascaris* worms from pigs in Iowa, USA, indicated that transmission had occurred from humans to pigs (Jesudoss Chelladurai et al. 2017). Some hybrid *Ascaris* have been found in humans from China, Guatemala, Europe, and Nepal, and among worms from pigs in China, Europe, and Guatemala (Zhou et al. 2012; Betson et al. 2014). Complete ITS2 nucleotide sequences of *Ascaris* samples from our study showed 100% similarity with *A. lumbricoides* from humans (e.g., GenBank no. AB571301) or *A. suum* from pigs (e.g., GenBank no. AB571302).

The phylogenetic tree constructed from *cox1* gene sequences showed three clusters (A, B, and C) that were the same as those depicted in the median-joining network (Fig. 3). Many haplotypes in these clusters have a wide distribution, suggesting frequent movement of *Ascaris* species around the world. This could be via infected people, or possibly pigs. Interestingly, seven new haplotypes were found, suggesting that further genetic diversity remains to be discovered in *Ascaris*. One new haplotype, from Lao PDR (haplotype 97), was grouped in cluster B with

*Ascaris* spp. from chimpanzee and gibbon. The remaining six of our new haplotypes were in cluster A with *Ascaris* spp. from human in Thailand and Lao PDR. This new diversity is another piece of the evolutionary jigsaw of *Ascaris*: such population markers are informative in monitoring worm dynamics during ongoing control efforts (Betson et al. 2011; Sparks et al. 2015). Cluster C was regarded by Nejsum et al. (2017b) as an early-diverging mitochondrial lineage, which has only been identified in pigs and humans from Slovakia, United Kingdom, Denmark, Italy, Uganda, and Tanzania. Probable hybrid *Ascaris* from Thailand (*cox1* haplotypes H27 and H58) as well as many *Ascaris* samples from Thailand, Lao PDR, and Myanmar fell into *cox1* cluster A. In the USA, 32% of *Ascaris* hybrids were in cluster A (Jesudoss Chelladurai et al. 2017). Moreover, *Ascaris* spp. from humans and pigs in Brazil shared common haplotypes in two widely separated geographical regions (I niguez et al. 2012). *Cox1* sequences from these probable hybrids exhibited haplotypes 27 and 58 (Fig. 3 and Supplemental Table S1), haplotypes that are known to occur in samples from pigs and from humans. The taxonomic status of both species remains contentious: do *A. lumbricoides* and *A. suum* constitute two species or one (Leles et al. 2012; Betson and Stothard 2016; da Silva Alves et al. 2016)?

On a cautionary note, we must point out that the present study used a pool of *Ascaris* eggs from each host for DNA extraction and sequencing. So, it is possible that eggs may have come from different female worms, perhaps representing both pure *A. lumbricoides* and *A. suum*. This possibility needs to be tested empirically in the future.

## Conclusions

To our knowledge, our study is the first molecular identification at the species level of *Ascaris* in Thailand, Lao PDR, and Myanmar. Also, this is the first report of probable hybrids between *A. suum* and *A. lumbricoides* in the region. Thus, zoonotic cross-transmission between humans and pigs, as revealed in Chiang Mai Province, northern Thailand, may occur. This is particularly likely at pig farms, where people are in frequent contact with pigs and use their manure as fertilizer. Farmers in this part of Thailand need to be made aware of hygiene precautions when handling pigs or their manure.

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## Compliance with ethical standards

**Ethical approval** This study was approved by the Human Ethics Committee of Khon Kaen University (Reference no. HE591403). Patient anonymity was maintained. Specimens of *Ascaris* adult worms from pigs obtained from local slaughterhouses do not correspond to the criteria for observational and field studies, and therefore, a separate ethics clearance was not required for these. No vertebrate specimens were used.

**Conflict of interest** The authors declare that they have no conflict of interest.

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