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Molecular Characterization of *Haemonchus contortus* (Nematoda: Trichostrongylidae) from Small Ruminants in Thailand Based on the Second Internal Transcribed Spacer of Ribosomal DNA

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ABSTRACT

The genetic variation of *Haemonchus contortus* in Thailand was studied including its relationship with Thai samples and relevant sequences. The second internal transcribed spacer (ITS2) of the rDNA sequence of *H. contortus,* a large stomach worm of small ruminants, was amplified and sequenced from 158 individual worms isolated from small ruminants (goats and sheep) distributed over 11 provinces in Thailand—Chiang Mai, Mae Hong Son, Krabi, Prachuap Khiri Khan, Buriram, Chaiyaphum, Suphanburi, Phra Nakhon Si Ayutthaya, Saraburi, Kanchanaburi and Ratchaburi. The analysis of nucleotide sequences revealed 21 genotypes (G1–G21). G1 and G2, comprising 62 and 55 individual worms, respectively, were predominant in the study. The results showed that genotype diversity was relatively high, with 12 polymorphic sites over 231 bp detected. The pairwise sequence identity of *H. contortus* was 98.4%. Comparing these sequences with those of *H. contortus* and *H. placei* derived from the GenBank database provided average results of 98.5% and 96.9%, respectively. Phylogenetic investigations using the UPGMA method found that *H. contortus* from the 21 genotypes in Thai populations was divided into two groups—group A and a separate group B containing G5 and G13. On a global scale, all Thai genotypes and sequences from the GenBank database of *H. contortus* were also related and grouped into the same cluster, The outgroups (*H. placei*) at both trees—the national and global scale were separated into a distinct cluster. The genetic information obtained in this study will be useful in many areas, including integrated approaches to issues such as farm management and anthelmintic drug treatment for haemonchosis infection.

Keywords: *Haemonchus contortus*, small ruminants, second internal transcribed spacer (ITS2) , genetic variation, Thailand

INTRODUCTION

Haemonchus contortus is commonly known as the barber pole worm belonging to the family Trichostrongylidae (Lichtenfels *et al*., 1994; Garretson *et al*., 2009; Gharamah *et al*., 2012), which is a gastrointestinal nematode found in the abomasum of goats and sheep (Roos *et al*., 2004), cattle (Akkari *et al*., 2013) and wild ruminants with a worldwide distribution (Cerutti *et al*., 2010). *H.*

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contortus is an important nematode economically and from veterinary studies is recognized to be one of the most serious diseases (known as haemonchosis) of sheep and goats (Hildreth and Harmon, 2013). This parasite also causes anemia together with edema because it feeds on blood, with symptoms including malnutrition and diarrhea and in heavy infections, severe anemia is due to the high parasite burden which can result in sudden death (Gasser *et al*., 2008; Garretson *et al*., 2009). In addition, *H. contortus* in goats and sheep is often able to resist many classes of anthelminthic drugs such as benzimidazoles, macrocyclic lactones, levamisole and imidazothiazoles (Roos *et al*., 1995; Papadopoulos *et al*., 2012). Generally, control of parasitic nematodes in livestock focuses on the use of anthelmintic drugs and farm management (Gasser *et al*., 2008). However, the knowledge derived from genetic information regarding this parasite such as genetic diversity and genetic relationships via phylogenetic trees is necessary to increase understanding of the ecology, epidemiology and evolution of parasitic nematodes (Jacquiet *et al*., 1995), and also to increase the efficacy of control programs. Since the knowledge of the genetic characterization of *H. contortus* in Thailand is still limited, there is a need to investigate the genetic diversity of this species to improve the control strategies for *Haemonchus* spp. infection. The intraspecific genetic variation of the second transcribed spacer (ITS2) sequences is relatively low (below 1%) according to Gasser and Newton (2000). The ITS2 sequences can be used as a tool for species differentiation such as in trematodes (Luton *et al*., 1992), cestodes (Králová-Hromadová *et al*., 2012) and gastrointestinal nematodes (Stevenson *et al*., 1995). Thus, the aim of this research was to study the genetic variation of ITS2 from *H. contortus* obtained from small ruminants in different locations in Thailand, and to investigate the genetic relationships among Thai samples and the sequences derived from the GenBank database (URL: http://www.ncbi.nlm.nih.gov/). This is the first study using ITS2 sequences of ribosomal DNA from *H. contortus* in Thailand and should provide knowledge on the basic genetic information for this parasite that can then be used in control programs for this parasitic nematode.

MATERIALS AND METHODS

Study area and parasite collection

Worms were collected from the abomasa of small ruminants (goats and sheep) from late 2012 to early 2013 from different regions in Thailand (11 provinces) as shown in Figure 1. These areas of study were selected depending on the available data on animal production from the website of the Department of Livestock Development (DLD)—namely, the provinces of Chiang Mai (CMI), Mae Hong Son (MSN), Krabi (KBI), Prachuap Khiri Khan (PKN), Buriram (BRM), Chaiyaphum (CPM), Suphanburi (SPB), Phra Nakhon Si Ayutthaya (AYA), Saraburi (SRI), Kanchanaburi (KRI), and Ratchaburi (RBR). However, data from Prachuap Khiri Khan were clustered in southern Thailand. The worms were obtained from the abomasa of 100 small ruminants (82 goats and 18 sheep) from different abattoirs in Thai-Muslim communities.

All worms from individual hosts were washed in 0.85% physiological saline and then preserved in 70% ethanol and stored at -20 °C until used. Only male worms were used in this study (Figure 2). Ten adult male worms of *H. contortus* obtained from each host within the same population were selected randomly and pooled together. Individual male worms were chosen from the pooled sample of each population and the anterior and posterior ends of each worm were cut away. The posterior end (Figure 2e) was used for species identification through spicule measurement under a microscope according to Jacquiet *et al.* (1997) and Achi *et al*. (2003), while the anterior end was employed for DNA extraction. *H. contortus* individuals of each population ranging from 10 to 17 (Table 1) were used for DNA study, after the species had been identified.

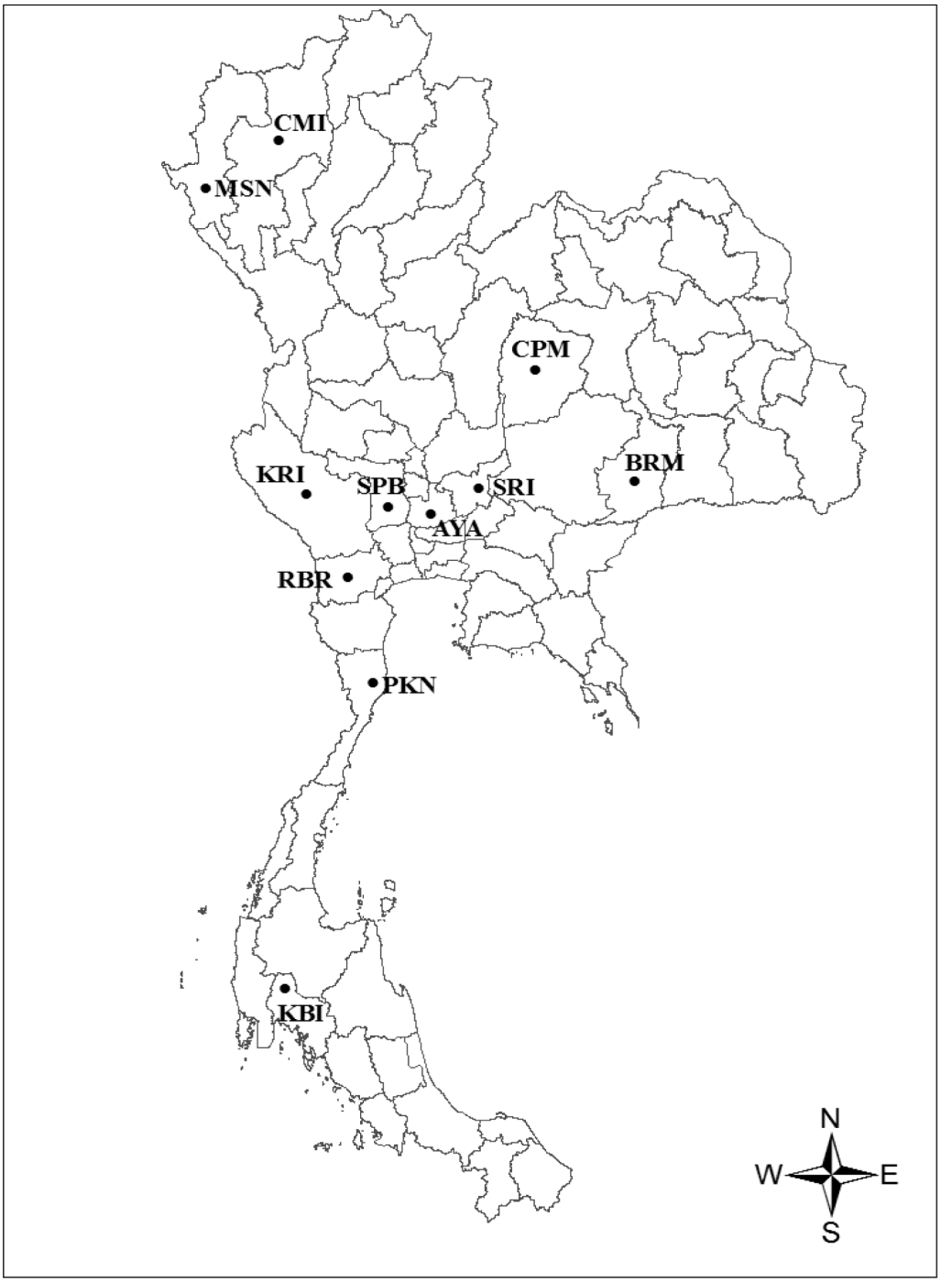

Figure 1 Sample locations of *H. contortus* populations in Thailand (CMI = Chiang Mai, MSN = Mae Hong Son, KBI = Krabi, PKN = Prachuap Khiri Khan, BRM = Buriram, CPM = Chaiyaphum, KRI = Kanchanaburi, RBR = Ratchaburi, SPB = Suphanburi, AYA = Phra Nakhon Si Ayutthaya, SRI = Saraburi).

In this study, 231 bp of 57 ITS2 sequences of *H. contortus* and 2 outgroups (*H. placei*) from the GenBank database were compared with Thai genotypes and the accession numbers and codes are shown in Table 2. There were no gaps presented over the alignment of 231 bp in any of the selected sequences. In total, these chosen sequences came from 14 countries in total: China (19 sequences), Lao PDR (1 sequence), Turkey (1 sequence), Uzbekistan (1 sequence), Iran (1 sequence), Japan (2 sequences), Australia (6 sequences), New Zealand (2 sequences), Tunisia (5 sequences), Czech Republic (2 sequences), France (1 sequence) and the USA (15 sequences), with one sequence of *H.contortus* studied from samples in different areas (Australia, United Kingdom, Switzerland and China), while 2 outgroups (X78812 and AJ577466 derived from Australia and France, respectively) were also included.

Genomic DNA isolation

After the species had been correctly identified from 158 individual nematodes, the worms stored at -20 °C were washed in 200 µL of ultrapure water (PURELAB® Ultra, ELGA LabWater/VWS Ltd.; Marlow, UK). Total genomic DNA was extracted from the anterior part of the 158 individual worms using a genomic DNA mini kit (tissue) following the manufacturer's instructions (Geneaid Biotech Ltd.; New Taipei City, Taiwan). The DNA was stored at -20 °C until used.

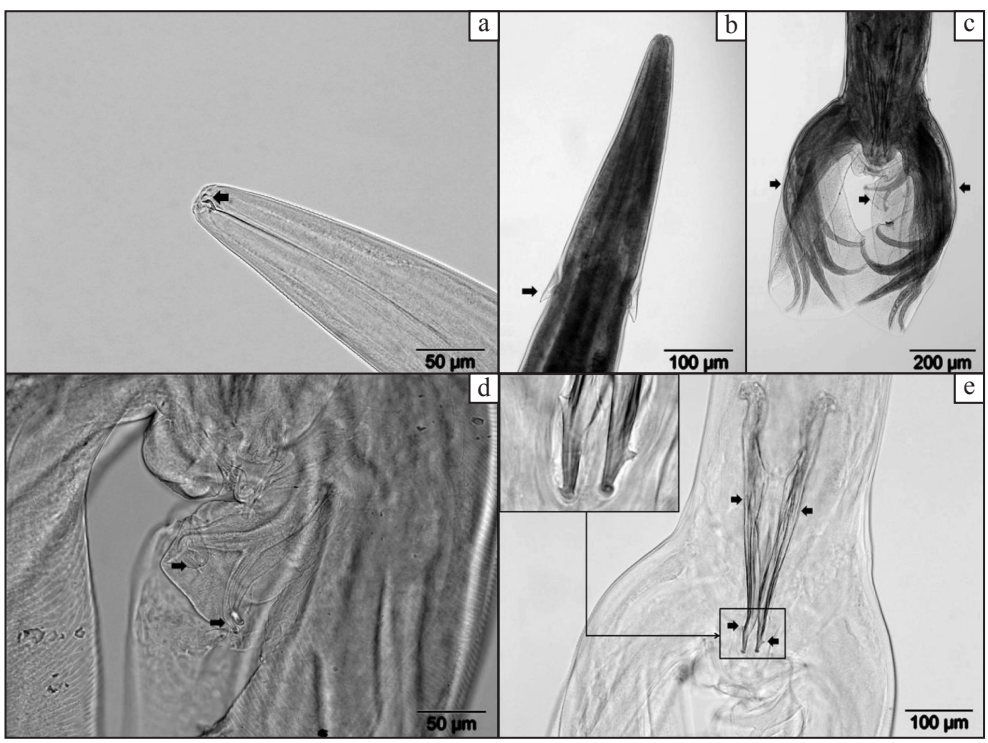

Figure 2 Morphometrics of *H. contortus* (male) from small ruminants: (a) Small buccal cavity with a single dorsal lancet (arrow); (b) Cervical papillae from the anterior end (arrow); (c) Bursa with two large lateral lobes (arrows at lateral lobes of bursa) and small asymmetrical dorsal lobe with Y shape dorsal ray (arrow inside the bursa); (d) Dorsal ray is bifurcated (arrows); (e) Each spicule (arrows outside the inserted box) with a single barb from its distal tip (arrows in the inserted box).

Table 1 Molecular diversity indices of second internal transcribed spacer gene of *H. contortus* populations in Thailand.

Population	North		South		Northeast		West			Central			Total
	CMI_Goats	MSN_Goats	KBI_Goats	PKN_Goats	BRM_Goats	CPM_Goats	KRI_Goats	RBR_Goats	KRI_Sheep	SPB_Goats	AYA_Sheep	SRI_Sheep	
Number of sequences	12	14	14	14	15	17	13	12	15	12	10	10	158
Number of genotypes	4	4	7	5	7	5	4	4	8	6	4	3	21
AT-rich (%)	67.07	67.13	67.10	67.10	66.98	67.1	67.10	67.06	67.13	67.05	67.10	67.10	67.09
GC-rich (%)	32.93	32.87	32.90	32.90	33.02	32.90	32.90	32.94	32.87	32.95	32.90	32.90	32.91
Number of polymorphic sites	2	4	10	6	9	5	4	5	10	4	5	3	12
Number of parsimony informative sites	1	1	8	4	7	2	1	4	5	2	1	1	8
Genotype diversity (SD)	0.742(0.084)	0.626(0.109)	0.846(0.074)	0.659(0.123)	0.857(0.065)	0.662(0.094)	0.679(0.089)	0.697(0.090)	0.848(0.071)	0.758(0.122)	0.711(0.118)	0.600(0.131)	0.724(0.025)
Nucleotide diversity (SD)	0.003(0.003)	0.004(0.003)	0.012(0.008)	0.007(0.005)	0.011(0.007)	0.005(0.004)	0.004(0.003)	0.007(0.005)	0.009(0.006)	0.005(0.004)	0.006(0.004)	0.004(0.003)	0.007(0.004)
Number of observed transitions	0	1	5	3	4	1	2	2	4	2	1	0	6
Number of observed transversions	2	3	6	3	5	4	2	3	6	2	4	3	7
Number. of substitutions (mutations)	2	4	11	6	9	5	4	5	10	4	5	3	13

Locations: CMI = Chiang Mai, MSN = Mae Hong Son, KBI = Krabi, PKN = Prachuap Khiri Khan, BRM = Buriram, CPM = Chaiyaphum, KRI = Kanchanaburi, RBR = Ratchaburi, SPB = Supanburi, AYA = Phra Nakhon Si Ayuthaya, SRI =Saraburi.

Second internal transcribed spacer sequence amplification

Polymerase chain reaction (PCR) products, 321 bp containing 231 bp of ITS2 plus franking sequence (20 bases from the 3' end of the 5.8S gene and 70 bp from the 5' end of the 28S gene) were amplified by PCR from the genomic DNA of individual worms using the conserved oligonucleotide primers of NC1 (forward 5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3') according to Stevenson *et al.* (1995). The PCR was carried out in the final volume of 20 µL with a final concentration of: 1X *Taq* buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% (volume per volume, v/v) Tween 20), 0.2 mM each of deoxynucleotide triphosphate (dNTP), 3 mM $MgCl_2$, 0.1 µM of each primer, 2U of *Taq* DNA polymerase (recombinant) sourced from Thermo Fisher Scientific Inc.; Waltham, MA, USA, and 2 µL of DNA template. The PCR conditions were performed in an automated thermocycle (Bio-Rad Laboratories; Hercules, CA, USA) with pre-denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min with a final extension at 72 °C for 5 min (Gharamah *et al*., 2012). A negative control (no DNA) was added into each experiment. PCR products with a length of 321 bp (Figure 3) were visualized on 1% agarose gel, stained with ethidium bromide and photographed using a Gel Doc system (Syngene, A Division of Synoptics Ltd.; Cambridge, UK).

DNA purification and sequencing

ITS2 products from the 158 adult male *H. contortus* worms were purified and sequenced by the Macrogen company (Seoul, Korea). Briefly, the double-stand PCR products were purified using the ExoSAP-IT PCR product cleanup as recommended by the manufacturer (USB® products, Affymetrix, Inc.; Cleveland, OH, USA) before the DNA samples were sequenced.

Table 2 Second internal transcribed spacer sequences of *H. contortus* from Thai genotypes and GenBank database from different geographical origins used in this study.

Accession Number	Code	Genotype/Isolate / Clone	Host	Stage	Geographical origin	Reference
KP101363	HcTh1	G1	Goats/sheep	Adult	SPB, SRI, AYA, KRI, RBR, CPM CMI, MSN, KBI, PKN, BRM/Thailand	Trop. Med., MU. Trop. Med., MU.
KP101364	HcTh2	G2	Goats/sheep	Adult	SPB, SRI, AYA, KRI, RBR, CPM CMI, MSN, KBI, PKN, BRM/Thailand	Trop. Med., MU. Trop. Med., MU.
KP101365	HcTh3	G3	Goats/sheep	Adult	PKN, BRM, CPM, KRI/ Thailand	Trop. Med., MU.
KP101366	HcTh4	G4	Goats	Adult	MSN, KBI,BRM, KRI(goat),SPB,Thailand	Trop. Med., MU.
KP101367	HcTh5	G5	Goats/sheep	Adult	KBI, BRM, KRI(sheep)/ Thailand	Trop. Med., MU.
KP101368	HcTh6	G6	Goats	Adult	SPB, CMI/Thailand	Trop. Med., MU.
KP101369	HcTh7	G7	Goats/sheep	Adult	MSN, PKN, SRI/Thailand	Trop. Med., MU.
KP101370	HcTh8	G8	Goats	Adult	KBI/Thailand	Trop. Med., MU.
KP101371	HcTh9	G9	Sheep	Adult	KRI, AYA/Thailand	Trop. Med., MU.
KP101372	HcTh10	G10	Goats	Adult	SPB, RBR/Thailand	Trop. Med., MU.
KP101373	HcTh11	G11	Goats/sheep	Adult	CMI, KRI(sheep), CPM/ Thailand	Trop. Med., MU.
KP101374	HcTh12	G12	Goats	Adult	SPB, BRM/Thailand	Trop. Med., MU.
KP101375	HcTh13	G13	Goats	Adult	KBI/Thailand	Trop. Med., MU.
KP101376	HcTh14	G14	Goats	Adult	PKN/Thailand	Trop. Med., MU.
KP101377	HcTh15	G15	Goats	Adult	RBR/Thailand	Trop. Med., MU.
KP101378	HcTh16	G16	Sheep	Adult	KRI/Thailand	Trop. Med., MU.
KP101379	HcTh17	G17	Goats	Adult	BRM/Thailand	Trop. Med., MU.
KP101380	HcTh18	G18	Goats	Adult	KBI/Thailand	Trop. Med., MU.
KP101381	HcTh19	G19	Sheep	Adult	AYA/Thailand	Trop. Med., MU.
KP101382	HcTh20	G20	Sheep	Adult	KRI/Thailand	Trop. Med., MU.
KP101383	HcTh21	G21	Goats	Adult	CPM/Thailand	Trop. Med., MU.
KC415117	HcCh1	Hlj10	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415118	HcCh2	Ln9	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415119	HcCh3	Sz9	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415120	HcCh4	Sz13	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415121	HcCh5	Yd2	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415122	HcCh6	Yn16	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415123	HcCh7	Yd14	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415124	HcCh8	Sz18	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415125	HcCh9	Sz22	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415126	HcCh10	Sz21	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415127	HcCh11	Sx7	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415128	HcCh12	Yd7	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415129	HcCh13	Sz1	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415130	HcCh14	Sz12	Goats/Sheep	Adult	China	Yin *et al*., 2013

Table 2 Continued

Accession Number	Code	Genotype/Isolate / Clone	Host	Stage	Geographical origin	Reference
KC415131	HcCh15	Ln1	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415132	HcCh16	Gx3	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415133	HcCh17	Yn20	Goats/Sheep	Adult	China	Yin *et al*., 2013
KC415134	HcCh18	Gx7	Goats/Sheep	Adult	China	Yin *et al*., 2013
HQ844231	HcCh19	Unknown	Sheep	Unknown	China	-
AB908961	HcLa1	isolate 1	*Capra hircus* (Goat)	Adult	Lao PDR	Otake *et al*., 2014
KJ188206	HcTk1	TrERUHcon07	Sheep	Egg	Turkey	-
KC503915	HcUz1	Unknown	*Ovis aries* (Sheep)	Unknown	Uzbekistan	Abramatov *et al*., 2013
HQ389229	HcIr1	Unknown	Sheep	Unknown	Iran	Nabavi *et al*., 2014
AB682686	HcJa1	Unknown	*Ovis aries* (Sheep)	Adult	Japan	Sultan *et al*., 2014
AB682687	HcJa2	Unknown	*Ovis aries* (Sheep)	Adult	Japan	Sultan *et al*., 2014
KF364628	HcAu1	Genotype F	Cattle	Adult and Egg	Australia	Jabbar *et al*., 2014
KF364629	HcAu2	Genotype G	Cattle	Adult	Australia	Jabbar *et al*., 2014
KF364630	HcAu3	Genotype H	Cattle	Adult	Australia	Jabbar *et al*., 2014
KF364631	HcAu4	Genotype I	Cattle	Adult	Australia	Jabbar *et al*., 2014
KF364632	HcAu5	Genotype J	Cattle	Adult	Australia	Jabbar *et al*., 2014
KC632567	HcAu6	HS123	*Homo sapiens*	Unknown	Australia	Koehler *et al*., 2013
X78803	HcSt1	Hc1-8	Sheep	Egg, L3 larvae and Adult	Australia (5) Switzerland (1), United kingdom(1) China (1)	Stevenson *et al.,* 1995
KC998713	HcNz1	Field variant 1	Sheep	Larvae	New Zealand	Bisset *et al*., 2014
KC998714	HcNz2	Field variant 2	Sheep	Larvae	New Zealand	Bisset *et al*., 2014
JX901144	HcTu1	Clone cattle1	Cattle	Adult	Tunisia	Akkari *et al.*, 2013
JX901154	HcTu2	Clone sheep4	Sheep	Adult	Tunisia	Akkari *et al.*, 2013
JX901156	HcTu3	Clone sheep1	Sheep	Adult	Tunisia	Akkari *et al.*, 2013
JX901157	HcTu4	Clone sheep2	Sheep	Adult	Tunisia	Akkari *et al.*, 2013
JX901158	HcTu5	Clone sheep3	Sheep	Adult	Tunisia	Akkari *et al.*, 2013
JX869075	HcCr1	Isolate 61	Sheep	Adult	Czech Republic	-
JX869073	HcCr2	Isolate 50	Sheep	Adult	Czech Republic	-
AJ577465	HcFr1	Unknown	Unknown	Unknown	France	-
EU084683	HcUs1	pDNA 5	Sheep	L3 larvae	USA	Garretson *et al*., 2009
EU084684	HcUs2	pDNA 13	Sheep	L3 larvae	USA	Garretson *et al*., 2009
EU084686	HcUs3	pDNA 23	sheep	L3 larvae	USA	Garretson *et al*., 2009
EU084687	HcUs4	pDNA 2	Goat	L3 larvae	USA	Garretson *et al*., 2009
EU084688	HcUs5	pDNA 12	Goat	L3 larvae	USA	Garretson *et al*., 2009
EU084689	HcUs6	pDNA 15	Goat	L3 larvae	USA	Garretson *et al*., 2009
EU084691	HcUs7	pDNA 22	Goat	L3 larvae	USA	Garretson *et al*., 2009
EU086378	HcUs8	GRF, clone 7	Giraffe	L3 larvae	USA	Garretson *et al*., 2009
EU086381	HcUs9	H992, clone 9	Sheep and Goats	L3 larvae	USA	Garretson *et al*., 2009

Table 2 Continued

Accession Number	Code	Genotype/Isolate / Clone	Host	Stage	Geographical origin	Reference
EU086382	HcUs10	H992, clone 10	Sheep and Goats	L3 larvae	USA	Garretson *et al*., 2009
EU086383	HcUs11	H992, clone 11	Sheep and Goats	L3 larvae	USA	Garretson *et al*., 2009
EU086385	HcUs12	H992, clone 14	Sheep and Goats	L3 larvae	USA	Garretson *et al*., 2009
EU086387	HcUs13	OS, clone 15	Sheep and Goats	L3 larvae	USA	Garretson *et al*., 2009
EU086390	HcUs14	RFR, clone 10	Sheep and Goats	L3 larvae	USA	Garretson *et al*., 2009
EU086393	HcUs15	RFR, clone 20	Sheep and Goats	L3 larvae	USA	Garretson *et al*., 2009
X78812	HpOg1	Hp1-3	Cattle	L3 and adult	Australia	Stevenson *et al*., 1995
AJ577466	HpOg2	Unknown	Unknown	Unknown	France	-

L3 larvae = Third stage larvae.

HpOg1 and HpOg2 = Outgroups (*H. placei*).

Trop. Med., MU. = Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

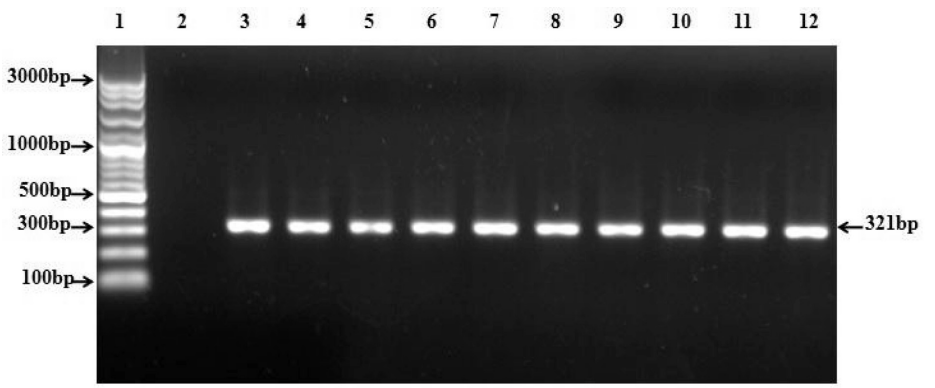

Figure 3 Polymerase chain reaction products (321 bp) including 231 bp of ITS2 gene of individual *H. contortus* (Lane 3-12). Lane 1 represents DNA marker (Vivantis Technologies: Selangar Darul Ehsan, Malaysia), Lane 2 represents negative control (no DNA).

The sequencing reactions were carried out in the DNA Engine Tetrad® 2 Peltier Thermal Cycler (Bio-Rad Laboratories; Hercules, CA, USA using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems; Foster City, CA, USA) with selected primers. Then, PCR products that were purified with the BigDye® XTerminator™ purification kit (Applied Biosystems; Foster City, CA, USA) were sequenced in both directions using an ABI 3730xl DNA analyzer (Applied Biosystems; Foster City, CA, USA).

Data analysis

All 158 *H. contortus* sequences from the 12 Thai populations were edited using MEGA software version 5 (Tamura *et al*., 2011) in both directions (forward and reverse sequences). All nucleotide sequences were compared with the sequences deposited in the GenBank database and the percentage similarity was observed via the BLAST server in the MEGA 5 software. The sequences were then aligned using Clustal W which is also integrated in the MEGA 5 program.

The pairwise sequence identity of Thai samples was computed using the BioEdit program (Hall, 1999) and compared with the reference sequences of *H. contortus* (accession numbers X78803 and EU084691) and *H. placei* (accession numbers X78812 and AJ577466).

The phylogenetic tree of the Thai populations and the global scale was constructed using the unweighted pair group method with arithmetic mean (UPGMA), distance method based on Kimura 2-parameter model (Kimura, 1980). All trees were constructed using the MEGA 5 program (Tamura *et al*., 2011) and two *H. placei* references (accession numbers X78812 and AJ577466) were added as outgroups.

The basic molecular parameters including the nucleotide diversity, genotype diversity, number of genotypes, number of polymorphic sites, number of parsimony informative sites and number of substitutions (transversion and transition) were computed using Arlequin version 3.5 (Excoffier and Lischer, 2011). DnaSP version 5 was used to compare some parameters (Librado and Rozas, 2009).

RESULTS

Basic genetic information of second internal transcribed spacer sequences

The 321 bp amplicons were amplified and sequenced from the 158 individual male worms of *H. contortus* collected from small ruminants (goats and sheep) in the 5 regions of 11 provinces in Thailand. All 231 bp of the ITS2 sequences were used in this study. In total, the 158 samples generated 21 genotypes: genotype 1 (G1) and genotype 2 (G2) were found to be the major genotypes and dominated in all areas studied (Table 3), accounting for 62 and 55 samples, respectively. The number of genotypes

Table 3 Number of *H. contortus* samples used in current study from different areas and genotypes in Thailand.

Region	Animal host	Locality	Number of specimens (total = 158)	Second internal transcribed spacer genotypes : G1–G21(number)
North	Goat	Chiang Mai (CMI)	12	G1(4), G2(5), G6(2), G11(1)
	Goat	Mae Hong Son (MSN)	14	G1(8), G2(4), G4(1), G7(1)
South	Goat	Krabi	14	G1(3), G2(5), G4(1), G5(1), G8(2), G13(1), G18(1)
	Goat	Prachuap Khiri Khan (PKN)	14	G1(8), G2(3), G3(1), G7(1), G14(1)
Northeast	Goat	Buriram (BRM)	15	G1(5), G2(3), G3(2), G4(1), G5(2), G12(1), G17(1)
		Chaiyaphum (CPM)	17	G1(9), G2(5), G3(1), G11(1), G21(1)
West	Goat	Kanchanaburi (KRI)	13	G1(6), G2(5), G3(1), G4(1)
		Ratchaburi (RBR)	12	G1(5), G2(5), G10(1), G15(1)
	Sheep	Kanchanaburi (KRI)	15	G1(4), G2(5), G3(1), G5(1), G9(1), G11(1), G16(1), G20(1)
Central	Goat	Suphanburi (SPB)	12	G1(2), G2(6), G4(1), G6(1), G10(1), G12(1)
	Sheep	Phra Nakhon Si Ayutthaya (AYA)	10	G1(5), G2(3), G9(1), G19(1)
		Saraburi (SRI)	10	G1(3), G2(6), G7(1)

Locations: CMI = Chiang Mai, MSN = Mae Hong Son, KBI = Krabi, PKN = Prachuap Khiri Khan, BRM = Buriram, CPM = Chaiyaphum, KRI = Kanchanaburi, RBR = Ratchaburi, SPB = Suphanburi, AYA = Phra Nakhon Si Ayutthaya, SRI = Saraburi.

in each population ranged from three to eight. The numbers of genotypes presented in Kanchanaburi (sheep), Krabi and Buriram provinces were higher than for other provinces, accounting for eight, seven and seven genotypes, respectively. The average AT and GC contents of the ITS2 sequences were 67.1% and 32.9%. For the alignment length of 231 bp of ITS2, there were 12 polymorphic sites at the alignment positions of 10, 18, 21, 22, 55, 59, 63, 78, 115, 123, 177 and 196 and there were 8 parsimony informative sites presented (Table 1 and 4).

Overall for the ITS2 molecular diversities, the genotype diversity and nucleotide diversity of the 21 genotypes of *H. contortus* from the 12 populations in Thailand were 0.724 ± 0.025 (ranging from 0.600 ± 0.131 to 0.848 ± 0.071) and 0.007 ± 0.004 (ranging from 0.003 ± 0.003 to 0.012 ± 0.008), respectively, (Table 1). Among the 12 polymorphic sites, seven positions were

Table 4 Polymorphic sites of the second internal transcribed spacer sequences from 21 genotypes of 158 *H. contortus* in different areas and two outgroups of *H. placei* with accession numbers X78812 (HpOg1) and AJ577466 (HpOg2). A dot indicates an identical nucleotide sequence with reference sequence G1. International Union of Pure and Applied Chemists nucleotide codes: A = Adenine, C = Cytosine, G = Guanine, T = Thymine, W = A or T.

	Variable positions of ITS2 sequence															
Genotypes	10	18	21	22	24	55	59	63	65	78	115	123	177	196	205	219
G1	C	T	G	T	A	C	T	C	A	T	T	T	G	T	G	A
G2	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
G3	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.	.
G4	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
G5	.	A	.	C	.	.	A	T	.	.	.	C	.	A	.	.
G6	.	.	.	.	.	.	.	.	.	.	.	.	.	W	.	.
G7	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.
G8	A	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
G9	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
G10	.	A	.	C	.	.	.	.	.	.	.	.	.	A	.	.
G11	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
G12	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
G13	.	A	.	C	.	.	G	T	.	C	.	C	.	A	.	.
G14	.	A	.	C	.	T	.	T	.	.	.	.	.	A	.	.
G15	.	A	.	C	.	T	.	.	.	.	.	.	.	A	.	.
G16	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.
G17	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.
G18	.	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.
G19	.	A	.	C	.	.	A	.	.	.	.	.	.	.	.	.
G20	.	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.
G21	A	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.
HpOg1	.	.	C	.	G	.	.	.	.	.	.	C	.	A	A	G
HpOg2	.	.	C	.	G	.	.	.	T	.	.	C	.	A	A	G

transversions (1: A<->C, 4: A<->T, 2: G<->C) while the remaining substitutions were transitions composed of six positions (5: T<->C, 1: A<->G). Of these substitutions, there was more than one different base substitution at position 59 with all transitions and transversions after data analysis. Moreover, the positions 24, 205, 219 and 65 are species-specific sequences for *H. placei* (Table 4).

The pairwise sequence identity among the 21 genotypes from the 158 individual Thai samples ranged from 96.1 to 99.5% (mean = 98.4%). These data were compared with the sequences of *H. contortus* and *H. placei* in terms of the identity obtained from GenBank database and the results showed the identity ranged from 96.5 to 100% (mean = 98.5%) and 96.1 to 98.7% (mean = 96.9%) for *H. contortus* and *H. placei,* respectively, (Table 5).

Phylogenetic analysis

The genetic relationship was reconstructed using the UPGMA method through the MEGA 5 program. The tree obtained showed 21 genotypes of *H. contortus* in Thailand which were divided into three main groups—A, B and outgroups (*H. placei*: X78812.1 and AJ577466.1) as shown in Figure 4.

Group A was composed of three subgroups—A1, A2 and A3. Subgroup A1 was made up of nine genotypes (G1, G3, G6, G7, G10, G11, G12, G17 and G19) while subgroup A2 consisted of eight genotypes (G2, G4, G8, G9, G16, G18, G20 and G21). Subgroup A3 comprised of G14 and G15. In Group B, there were two genotypes—G5 and G13. The outgroups were clearly separated from *H. contortus* in group A and B (Figure 4).

In the global study (Figure 5), 80 sequences of *H. contortus* including 2 outgroups produced a tree that was also divided into three major groups (A, B and outgroups). All genotypes of *H. contoutus* in Thailand were distributed within two groups. In group A, the Thai genotypes were related with the samples in all countries. However, eight Thai genotypes in this group presented 100% similarity with the sequences from several selected countries; for example, G2 (HcTh2) showed similarity with the sequences of eight countries—the USA (HcUs7, HcUs8, HcUs11 and HcUs15), Laos (HcLa1), New Zealand (HcNz2), Uzbekistan (HcUz1), China (HcCh1 and HcCh19), Turkey (HcTk1), Iran (HcIr1) and Australia (HcAu3 and HcAu6). G1 (HcTh1) showed sequence similarities with Australia (HcAu1), China (HcCh8) and the Czech Republic (HcCr1). The remaining six Thai genotypes were similar to six sequences from China and one sequence from Australia—HcTh7 (G7):HcCh17, HcTh11(G11):HcCh18, HcTh9(G9):HcCh6, HcTh12(G12):HcCh11:HcAu5, HcTh21(G21):HcCh5 and HcTh8(G8):HcCh7. In group B, the results revealed that some Thai and Chinese sequences were predominant and two pairs of sequences showed 100% similarity (HcTh10(G10):HcCh13 and HcTh3(G3):HcCh16). Similarly, two outgroups of *H. placei* with accession numbers X78812 (HpOg1) and AJ577466 (HpOg2) were also clearly distinguished from groups A and B.

DISCUSSION

The current study provided the first data of the ITS2 sequences derived from 158 *H. contortus* samples sourced from small ruminants (goats and sheep) in different areas of Thailand. ITS2 has been widely used and has become a useful tool for species identification because it is highly species specific, has more conservative regions and has universal primers binding to the 5.8S and 28S ribosomal DNA genes of several nematodes (Epe *et al*., 1997; Heise *et al*., 1999) and trematodes (Luton *et al*., 1992).

Analysis of the 158 individual sequences of the ITS2 gene from small ruminants located in 11 provinces in Thailand produced 21 genotypes. Two genotypes (G1 and G2) were generally found

Table 5 Pairwise sequence identities (%) within second internal transcribed spacer sequences of *H. contortus* representing 158 individual worms from different areas in Thailand compared with the sequences of *H. contortus* and *H. placei* from the GenBank database.

Genotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. G1																									
2. G2	99.5																								
3. G3	99.1	98.7																							
4. G4	99.5	99.1	98.7																						
5. G5	97.4	96.9	98.2	96.9																					
6. G6	99.5	99.1	98.7	99.1	97.4																				
7. G7	99.1	98.7	99.1	98.7	98.2	99.1																			
8. G8	99.1	99.5	98.2	98.7	96.5	98.7	98.2																		
9. G9	99.5	99.1	98.7	99.1	96.9	99.1	98.7	99.5																	
10. G10	98.7	98.2	99.5	98.2	98.7	98.7	99.5	97.8	98.2																
11. G11	99.5	99.1	99.5	99.1	97.8	99.1	99.5	98.7	99.1	99.1															
12. G12	99.5	99.1	99.5	99.1	97.8	99.1	98.7	98.7	99.1	99.1	99.1														
13. G13	96.9	96.5	97.8	96.5	99.1	96.9	97.8	96.1	96.5	98.2	97.4	97.4													
14. G14	97.8	97.4	98.7	98.2	98.7	97.8	98.7	96.9	97.4	99.1	98.2	98.2	98.2												
15. G15	98.2	97.8	99.1	98.7	98.2	98.2	99.1	97.4	97.8	99.5	98.7	98.7	97.8	99.5											
16. G16	99.1	98.7	98.2	99.5	96.5	98.7	98.2	98.2	98.7	97.8	98.7	98.7	96.1	97.8	98.2										
17. G17	99.1	98.7	98.2	98.7	97.4	99.1	99.1	98.2	98.7	98.7	98.7	98.7	96.9	97.8	98.2	98.2									
18. G18	99.1	99.5	98.2	99.5	96.5	98.7	98.2	99.1	98.7	97.8	98.7	98.7	96.1	97.8	98.2	99.1	98.2								
19. G19	98.7	98.2	99.5	98.2	98.7	98.2	98.7	97.8	98.2	99.1	99.1	99.1	97.8	98.2	98.7	97.8	97.8	97.8							
20. G20	99.1	98.7	98.2	99.5	97.4	99.1	99.1	98.2	98.7	98.7	98.7	98.7	96.9	98.7	99.1	99.1	99.1	99.1	97.8						
21. G21	98.7	99.1	97.8	98.2	96.9	98.7	98.7	99.5	99.1	98.2	98.2	98.2	96.5	97.4	97.8	97.8	98.7	98.7	97.4	98.7					
22. Hc:X78803	98.7	99.1	97.8	98.2	97.8	98.7	98.7	98.7	98.2	98.2	98.2	98.2	97.4	97.4	97.8	97.8	98.7	98.7	97.4	98.7	99.1				
23. Hc:EU084691	99.5	100	98.7	99.1	96.9	99.1	98.7	99.5	99.1	98.2	99.1	99.1	96.5	97.4	97.8	98.7	98.7	99.5	98.2	98.7	99.1	99.1			
24. Hp:X78812	97.4	97.8	96.5	96.9	96.5	97.4	97.4	97.4	96.9	96.9	96.9	96.9	96.1	96.1	96.5	96.5	97.4	97.4	96.1	97.4	97.8	98.7	97.8		
25. Hp:AJ577466	96.9	97.4	96.1	96.5	96.1	96.9	96.9	96.9	96.5	96.5	96.5	96.5	95.6	95.6	961	96.1	96.9	96.9	95.6	96.9	97.4	98.2	97.4	99.5	

Hc and Hp represent *H. contortus* and *H. placei* (outgroup), respectively

in all locations in Thailand. In a similar study in China, 152 individual worms obtained from small ruminants (goats and sheep) generated 18 genotypes distributed across 7 different locations (Yin *et al*., 2013). In another investigation, involving 92 individual worms of *Haemonchus* from experimentally infected sheep with worms that originated from cattle, 14 adult worms were identified as *H. contortus* with 5 genotypes designated as genotypes F, G, H, I and J (Jabbar *et al.,* 2014). Here, the GC content was 32.9% which was similar to some previous reports; for example, 33% reported from sheep in Australia, the United Kingdom, Switzerland and China (Stevenson *et al*., 1995), 33.4% from goats and sheep in Malaysia and Yemen, (Gharamah *et al*., 2012), 33% from sheep and cattle and 36% from goats in Tunisia (Akkari *et al*., 2013), about 33% from cattle in Australia (Jabbar *et al.,* 2014) and 33% from sheep in Uzbekistan (Abramatov *et al*., 2013). Moreover, the ITS2 sequences of *H. contortus* had a high number of polymorphic sites and substitutions of up to 12 and 13 positions (7 transversions and 6 transitions), respectively. The position 59 presented two bases (A and G) at one position. This finding concurred with Hoste *et al*. (1995), who detected two bases at one position in the ITS2 sequence of *Trichostrongylus axei* and five different positions in *T. vitrinus*. In addition, in Tunisia, Akkari *et al*. (2013) found 10 substitutions over the length of 231 bp from *H. contortus* in goats, while Gasser *et al*. (1998) found 12 nucleotide variations (4 transitions, 5 transversions, 1 insertion and 2 deletions) of ITS2

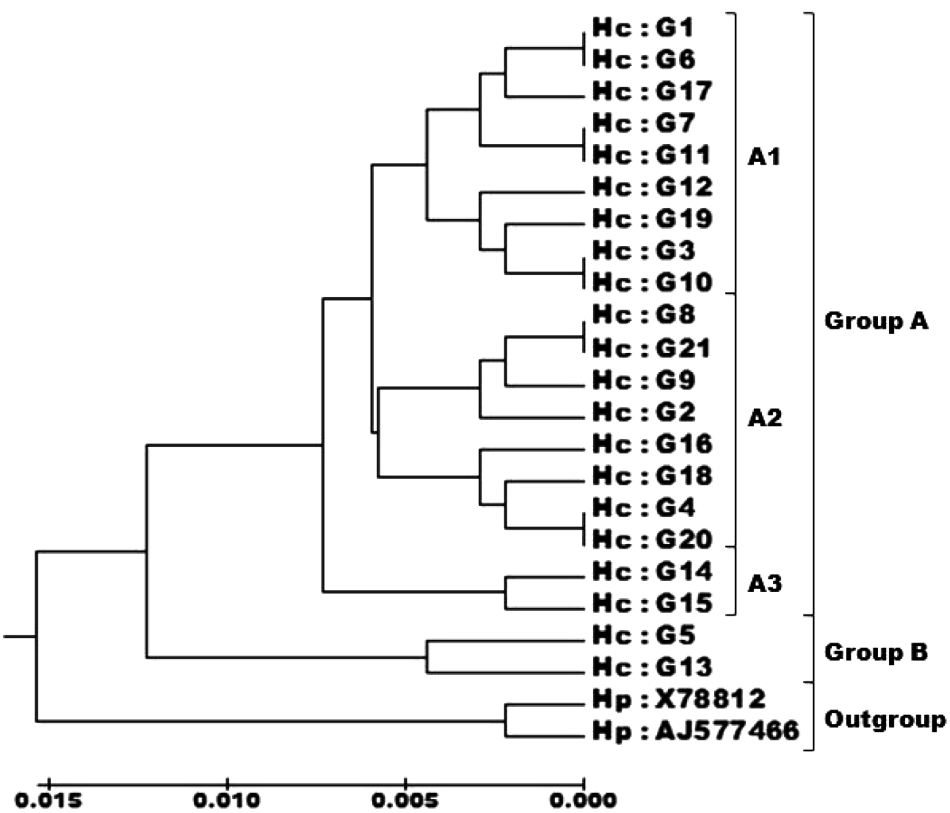

Figure 4 Unweighted pair group method with arithmetic mean dendrogram showing the relationships among *H. contortus* genotypes from small ruminants based on second internal transcribed spacer sequence data from different regions in Thailand with *H. placei* (GenBank accession numbers X78812 and AJ577466) as outgroup.

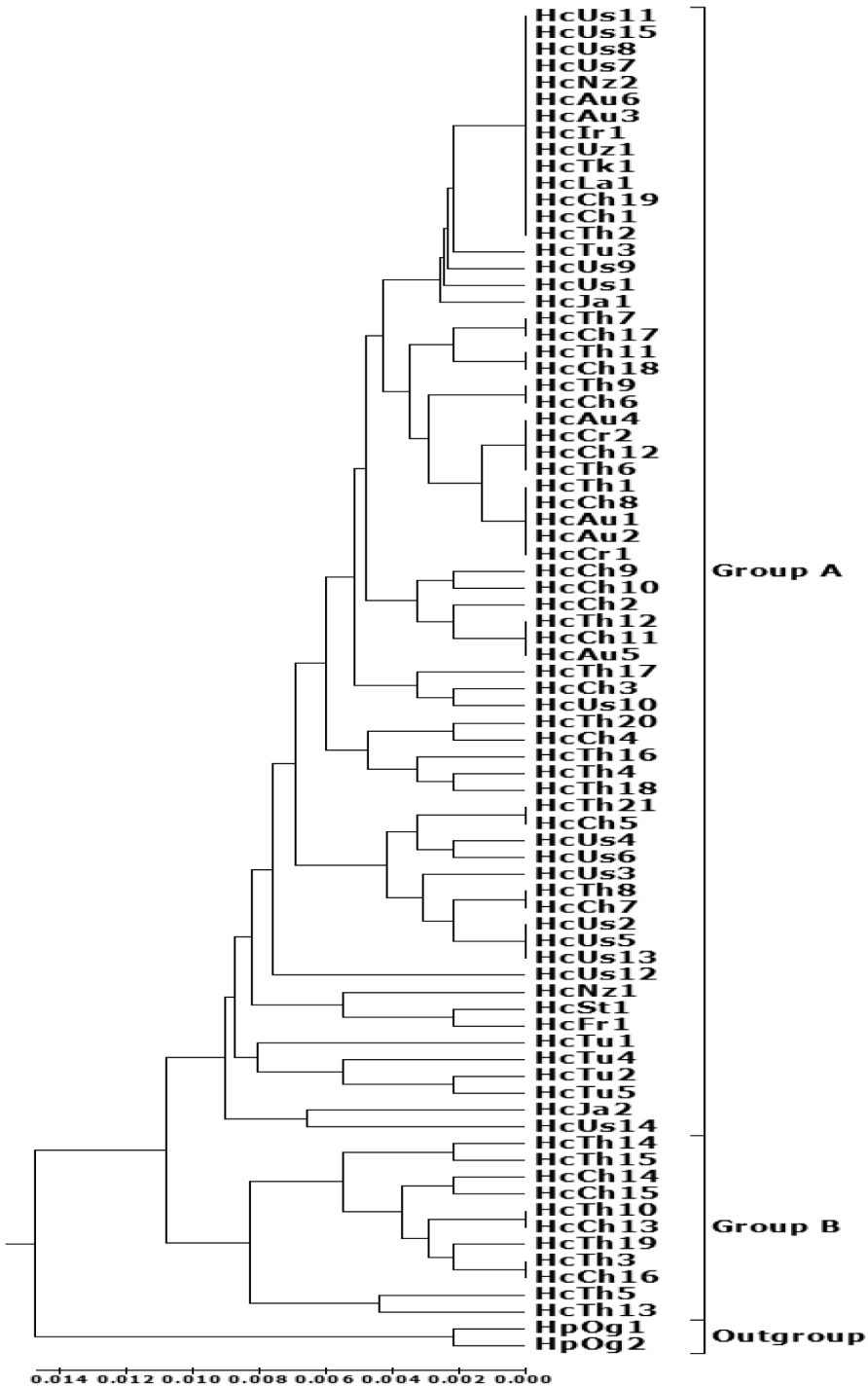

Figure 5 Unweighted pair group method with arithmetic mean dendrogram showing the relationships among *H. contortus* genotypes from small ruminants based on second internal transcribed spacer sequences from different regions in Thailand and other countries from GenBank database, with *H. placei* accession numbers X78812 (HpOg1) and AJ577466 (HpOg2) as outgroup.

in *H. contortus* samples in goats and sheep from various countries. In China, six substitutions of the ITS2 gene were also detected from this nematode in goats and sheep (Yin *et al*., 2013).

In the current study, the nucleotide diversity of ITS2 from *H. contortus* was low (0.007 ± 0.004), while the genotype diversity was quite high (0.724 ± 0.025). In China, the nucleotide and genotype diversity ranged from 0.0054 to 0.0084 and from 0.609 to 0.824, respectively (Yin *et al*., 2013). Similarly, a high diversity of ITS2 sequences in Tunisia was observed in *H. contortus* sequences (Akkari *et al*., 2013). The high genotype diversity in the current study might have been due to the parasites used coming from different individual hosts before being pooled together with those from the same area prior to analysis. The movement of animal hosts within the country and between some neighboring countries such as Lao PDR (Otake *et al*., 2014) or China (Yin *et al*., 2013) by humans, might also allow gene flow to occur.

The pairwise sequence identity among the 21 genotypes of *H. contortus* ranged from 96.5 to 100%, (mean = 98.5%, 1.5% intraspecific variation) when compared with *H. contortus* from the GenBank database, while pairwise identity between *H. contortus* and *H. placei* (two outgroups) ranged from 96.1 to 98.7%, (mean = 96.9%, 3.1% intraspecific variation). In China, the sequence identity of 18 genotypes among ITS2 sequences comparised with references (*H. contortus* and *H. placei* from Genbank) ranged from 97.4 to 100% (0–2.6% intraspecific variation) and from 96.1 to 98.2% (1.8–3.9% intraspecific variation), respectively, (Yin *et al*., 2013). In Tunisia, the sequence similarity of ITS2 *H. contortus* sequences from goats, sheep, and cattle showed 98% homology (2% intraspecific variation) according to Akkari *et al*. (2013). In Germany, the investigation of ITS2 from eight species of gastrointestinal nematodes reported the degree of intraspecific variation of *H. contortus* at 2.6% (Heise *et al*., 1999). Moreover, another species of nematode, *Dictyocaulus eckerti* or the lungworm nematode also showed 0.6–3.3% intraspecific variation (Epe *et al*., 1997). Normally, the ITS2 of nuclear ribosomal DNA has proven to be a useful and reliable genetic marker for species identification (Campbell *et al*., 1995; Stevenson *et al*., 1995; Gasser, 2006) because it is highly species specific (Heise *et al*., 1999) and shows low intraspecific variation at the species level. However, the genetic variability of ITS2 within individual organisms appeared to differ depending on the parasite population studied (Gasser and Newton, 2000; Gasser, 2006). Furthermore, Wesson *et al*. (1992) suggested that a polymorphism in the ITS2 appeared to be the consequence of mutational change occurring during DNA replication.

Three nucleotide positions showed species-specific sequences at positions 24, 205 and 219. These were found only in *H. placei* (accession numbers X78812 and AJ577466), with the bases corresponding to the positions at 24, 205 and 219 being G, A and G, respectively. Moreover, Stevenson *et al*. (1995) also discovered the position of recognition site of *Bfa*I at positions 21–24 (CTAG). This recognition site presented specifically only in *H. placei* and it was not found in any of the genotypes of *H. contortus* in this study (Table 4).

The phylogenetic relationships (Figures 4 and 5) showed that all 21 genotypes of *H. contortus* from the Thai samples were also related to each other both at the country and global level. However, G5 and G13 were scparated from other *H. contorius*. Interestingly, some results obtained from the UPGMA tree at the global scale showed that genotype 2 (HcTh2) in Thailand in group A (Figure 5) was similar to the sequences from Australia (HcAu6) and the USA (HcUs8 and HcUs15). HcAu6 was sequenced from *H. contortus* collected from stool samples of people in Australia (Koehler *et al*., 2013). Thus, this genotype might pose an infection threat for Thai people since the parasite may adapt to a new host.

Supporting evidence for this idea can be found in Ghadirian and Arfaa (1973), where *H. contortus* was detected in fecal samples of Iranians. The HcUs8 sequence was obtained from a parasite belonging to giraffes raised in a Florida zoological park in the USA (Garretson *et al*., 2009). Giraffes and other wildlife are recognized reservoir hosts (Haydon *et al*. 2002). In Thailand, giraffes in zoos have been imported from aboard. This importing of potential host individuals is one of several factors that cause the spread of diseases to ruminants in Thailand. Another interesting result was the genotype G2 (HcTh2) being related to *H. contortus* isolated in the USA (HcUs15), that has been continuously exposed to anthelmintic drugs (Garretson *et al*., 2009). This indicated that drug resistance in Thailand might occur in all areas studied. Normally, anthelminthic drugs are used in animal farms in Thailand (Pralomkarn *et al*., 2012).

CONCLUSION

This was the first study of the genetic variation in *H. contortus* sampled from small ruminants (goats and sheep) from different areas in Thailand. These results showed genotypic diversity which was quite high and demonstrated polymorphism. The common genotypes G1 and G2 were detected in 21 genotypes. Most of the sequences of this parasite showed genetic relationships to all other Thai and global samples. The exceptions were G5 and G13, which were quite different from the other genotypes when using trees constructed at both the national and global scale. The genetic information obtained in this study provides basic knowledge to better inform parasite control programs. It will also improve integrated approaches such as farm management and anthelmintic drug treatment. The findings also highlight potential future issues. Some genotypes such as G5 and G13 of *H. contortus* were identified from animals located in some provinces—namely, Krabi, Buriram and Kanchanaburi—where extra measures should be taken to ensure problems are avoided in the future.

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