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Original Article

Epidemiology of Helminthic Infections and Phylogenetic Tree of *Strongyloides stercoralis* in Rubber Tree Plantation in Lower Northern Part of Thailand

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Received 16 Nov 2024
Accepted 13 Jan 2025

Keywords:
Epidemiology;
Phylogenetic analysis;
Rubber tree plantation;
Strongyloides stercoralis

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Abstract

Background: Helminthic infections cause helminthiasis, including infections by *Strongyloides stercoralis*, a kind of helminths that cause reinfection and lead to severe infections, can be transmitted through the soil. We aimed to identify *S. stercoralis* and other helminthic infections in rubber tree plantations in Thailand's lower northern regions. The specific goals include assessing prevalence using Formalin Ethyl–acetate Concentration Technique (FECT) and Agar Plate Culture (APC) and constructing *S. stercoralis* phylogenetic tree.

Methods: Overall, 646 fecal samples from rubber plantation workers in five provinces in northern Thailand were examined using FECT and APC under microscope. DNA from larvae confirmed as *Strongyloides* spp. by Polymerase Chain Reaction (PCR) was sequenced for phylogenetic analysis. The DNA sequences were also submitted to the GenBank database.

Results: Prevalence of helminthic infections was 8.82%, with soil transmitted helminths (STH) prevalence at 6.81%; *S. stercoralis* accounted for 5.41%, with *Ascaris lumbricoides* at 0.62%, hookworm 0.46%, and *T. trichiura* 0.31%. PCR analysis successfully amplified the 18S rRNA gene in 26 out of 34 genomic DNAs, indicating a detection rate of 70.59%. Sequencing of these PCR products identified *S. stercoralis* strains closely related to those reported in the Republic of Lao, Myanmar, and Japan, suggesting genetic diversity within the species.

Conclusion: STH prevalence, predominantly *S. stercoralis*, highlights public health concerns in rubber plantation areas, necessitating enhanced monitoring and intervention strategies. Phylogenetic analysis of *S. stercoralis*, revealing a close genetic relationship among strains from various Southeast Asian countries, which underscores potential patterns of transmission and evolutionary relationships in the regions.

Introduction

In tropical regions worldwide, including Thailand, helminth infections continue to be a persistent problem. The most prevalent helminths parasites encountered worldwide are STH including hookworm, *Ascaris lumbricoides*, *Trichuris trichiura* and *Strongyloides stercoralis* in addition to foodborne helminth like *Opisthorchis viverrini*, *Taenia* spp. (1). The WHO reports that an estimated 1.5 billion infected people or 24% of the world's population, are infected with intestinal parasites infections, mainly the STH (2).

A study in southern Thailand revealed prevalence rates: *A. lumbricoides* (3.7-18.5 %), *T. trichiura* (28.5-66.9 %), hookworm (18.0-80.0 %), and *S. stercoralis* (0.3-20.6%) (3-4). Of these, *S. stercoralis* infections emerged as particularly grave, often leading to severe illness and high mortality rates, especially among immunocompromised individuals. Additionally, such infections can perpetuate in a vicious cycle of autoinfection, posing recurrent threats of severe illness (5).

S. stercoralis is a roundworm in the human intestine. This parasitic organism can be observed in various geographical locations across the globe, with a particular prevalence in regions ranging from tropical to temperate climates (6). The infection can be contracted through the skin from soil and subsequently spread to various parts of the body, resulting in the development of Strongyloidiasis. In Thailand and Lao PDR, the prevalence of *S. stercoralis* was 0.3-33.4% (3,4,7). The prevalence of high *S. stercoralis* infections in the southern region part of Thailand can be attributed to its hot and humid climate, as well as the extensive cultivation of rubber trees. These environmental conditions create a suitable habitat for soil-borne parasites to thrive. It is worth noting that individuals with poor hygiene in rural areas or those engaged in agricultural activities are more susceptible to contracting relatively high *S. stercoralis* infections. Southern Thailand is typically the main area for rubber plantations, and the planting areas experienced significant changes after the Thai government launched the rubber-planting project (8). Nowadays, the rubber plantation area has expanded to all regions of Thailand, including the northeastern and northern regions. It has also expanded throughout Southeast Asian (SEA) such Laos, Myanmar, Vietnam, Cambodia (9). Hence, the dissemination of STH and helminth infection can potentially occur in Thailand, SEA, and various regions worldwide.

The primary objective of this investigation is to identify *S. stercoralis* and other helminths, as well as construct a phylogenetic tree specifically for *S. stercoralis* within a rubber tree plantation located in the lower north part of Thailand. To achieve this, we employed FECT, APC and PCR. Epidemiological information on *S. stercoralis* and other helminths were obtained from this study. It will benefit basic public health in Thailand and around the world.

Materials and Methods

The study sites

The research was conducted in the lower northern region of Thailand across five provinces: Phitsanulok, Sukhothai, Phetchabun, Phichit, and Uthai Thani (Fig. 1).

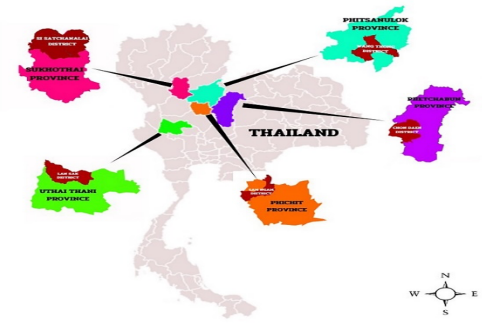

Fig. 1: Geographical map depicts the study area across five provinces situated within the lower northern region of Thailand

Specimen collection

A total of 646 fecal samples were gathered from rubber plantations in the lower northern region of Thailand. These samples were collected from five provinces, namely: Ban Klang Subdistrict, Wang Thong District, Phitsanulok Province (171 samples); Si Satchanalai District, Sukhothai Province (150 samples); Huay Nga Chang Subdistrict, Khandan District, Phetchabun Province (112 samples); Nong Sano Subdistrict, Sam Ngam District, Phichit Province (106 samples); and Mae Sin Subdistrict and Rabam Sub-district, Lan Sak District, Uthai Thani Province (107 samples). The fecal samples were then subjected to the Agar plate culture method and Formalin ethyl-acetate concentration techniques. They were subsequently divided into 1.5 micro centrifuge tubes and stored at -20 °C for PCR detection. The remaining feces were divided for detection using other methods. This research study underwent ethical review and received approval from the Human Ethics Committee, Naresuan University, Thailand. The project was assigned the code IRB No. 317/58 and was dated 18 September 2015.

APC and FECT methods

The stool samples were examined by APC and FECT under microscope. APC served to diagnose *S. stercoralis* and other parasites such as hookworm, whereas FECT was employed to detect other intestinal parasitic infections. Stool samples that tested positive for parasites using either of these laboratory techniques were categorized as positive.

Molecular characterization of S. stercoralis

The genomic DNA extraction analysis was use of the nucleospin tissue extraction kit from

Macherey-Nagel GmbH & Co., Duren, Germany. Initially, samples were carefully examined under a microscope, and a single *S. stercoralis* specimen was selected from each culture. This strict selection criterion was essential to ensure the accuracy of subsequent experimental results, thereby improving the reliability and validity of the findings. Only one *S. stercoralis* specimen was obtained from all samples before extraction for precise experimental results. A total of 34 samples were used for the experiment, including 12 samples from Phitsanulok, 11 from Sukhothai, 4 from Phetchabun, 4 from Phichit, and 3 from Uthai Thani Province. All 34 samples were subjected to PCR detection analysis. Following the extraction of DNA the primers for 18S ribosomal RNA – 568 base pairs were used. Forward primer MSP4F (5'-CGA AAG CAT TTG CCA AG-3') and Reverse primer StrongR (5'-AAC AGG AAC ATA ATG ATC ACT AC-3') were used for the amplification of the 18S rDNA (10). The PCR was carried out in 20 µL reaction, which consisted of Master mix 10 µL, 0.4 µL of each primer, 2 µL DNA template and 7.2 µL deionized water (18 MΩ). The PCR procedure commenced with an initial denaturation step at 94 ℃ for 2 minutes, followed by 30 cycles consisting of 30 seconds at 94 ℃, 30 seconds at 61.5 ℃, and 30 seconds at 72 ℃. A final extension phase at 72 ℃ for 10 minutes concluded the process. Subsequently, the PCR products underwent electrophoresis on a 1.2% agarose gel. Finally, the gel was stained using a nucleic acid staining solution (Red SafeTM, iNtRON Biotecnology, Inc.) for analysis. The visualization of a 568 bp was considered as a positive result.

Phylogenetic analysis

The PCR products were purified utilizing the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany), following the manufacturers protocol. DNA sequencing was conducted by Macrogen Inc. in Korea. Subsequently, gene sequences underwent analysis through a nucleotide BLAST search using the NCBI program Seqman II, followed by alignment using Clustal W. The obtained gene sequences were compared with definitive nucleotide sequences available in The National Center for Biotechnology Information. These sequences were then utilized to construct a phylogenetic tree employing the neighbor-Joining method via MEGA Version 7.0 software. Additionally, the DNA sequences were submitted to

the GenBank database and utilized in BLAST searches for further analysis.

Results

Prevalence of helminth infections Using APC and FECT

During our investigation into helminth parasite prevalence among rubber plantation workers in the lower-northern provinces of Thailand, a total of 646 stool samples were analyzed, revealing 57 cases of infection (8.82%). The prevalent helminth parasites include *S. stercoralis* (5.41%), *O. viverrini* (0.93%), *A. lumbricoides* (0.62%), *Taenia* spp. (0.62%), *T. trichiura* (0.31%), hookworm (0.31%), *Hymenolepis nana* (0.31%), and *Dipylidium caninum* (0.15%), as indicated in Table 1.

Table 1: Variety of prominent helminthic infections and their prevalence

Sample population (province)	Number of samples	Ss (%)	Ov (%)	Al (%)	Tsp (%)	Hw (%)	Tt (%)	Hn (%)	Dc (%)	Total (%)
Phitsanulok	171	12 (7.02)	4 (2.34)	3 (1.75)	2 (1.17)	1 (1.17)	2 (0.58)	2 (1.17)	1 (0.58)	27 (15.79)
Sukhothai	150	11 (7.33)	2 (1.33)	0	0	1 (0.66)	0	0	0	14 (9.33)
Phetchabun	112	5 (4.46)	0	0	1 (0.89)	1 (0.89)	0	0	0	7 (6.25)
Phichit	106	4 (3.78)	0	1 (0.94)	1 (0.94)	0	0	0	0	6 (5.66)
Uthai Thani	107	3 (2.80)	0	0	0	0	0	0	0	3 (2.80)
Total	646	35 (5.41)	6 (0.93)	4 (0.62)	4 (0.62)	3 (0.46)	2 (0.31)	2 (0.31)	1 (0.15)	57 (8.82)

Ss=*S. stercoralis*, Ov= *O. viverrini*, Al= *A. lumbricoides*, Tsp= *Taenia* spp, Hw= hookworm, Tt= *T. trichiura*, Hn= *H. nana*, Dc= *D. caninum*

Within the rubber plantations spanning Thailand's lower-northern region, we observed the prevalence of STH. Across the five provinces examined, 44 infections (6.81%) were documented, with the breakdown of specific parasites as follows: *S. stercoralis* at 5.41%, *A. lumbricoides* at 0.62%, hookworm at 0.46%, and *T. trichiura* at 0.31%. The prevalence of infection cases in each province within the region is outlined as follows: Phitsanulok reported a prevalence rate of 10.53%, Sukhothai at 8.00%, Phetchabun at 5.36%, Phichit at 4.72%, and Uthai Thani at 2.82% (Table 2).

Table 2: Prevalence of STH detected with APC and FECT

Population Sample (province)	Number of samples	Ss (%)	Al (%)	Hw (%)	Tt (%)	Total (%)
Phitsanulok	171	12 (7.02)	3 (1.75)	1 (1.17)	2 (0.58)	18 (10.53)
Sukhothai	150	11 (7.33)	0	1 (0.66)	0	12 (8.0)
Phetchabun	112	5 (4.46)	0	1 (0.89)	0	6 (5.36)
Phichit	106	4 (3.78)	1 (0.94)	0	0	5 (4.72)
Uthai Thani	107	3 (2.80)	0	0	0	3 (2.82)
Total	646	35 (5.41)	4 (0.62)	3 (0.46)	2 (0.31)	44 (6.81)

To assess and establish the prevalence of parasites, we conducted a comparative analysis of two techniques: the FECT and APC. Among the 646 fecal samples examined, the FECT method revealed a total prevalence of 5.73%, detecting eight parasite types. These included *S. stercoralis, O. viverrini, A. lumbricoides, Taenia* spp., hookworm, *T. trichiura, H. nana, and D. caninum*. In contrast, the agar plate culture technique could detect only *S. stercoralis* but exhibited notably higher prevalence rates for *S. stercoralis* (5.26% comparing to 2.32% with FECT), as illustrated in Table 3. While 19 additional samples (2.94%) were exclusively detectable through the Agar plate culture technique, underscoring its superior capability for this parasite, it is noteworthy that one sample (0.15%) was solely identifiable by FECT.

Table 3: Comparative analysis of helminthic detection methods: FECT and APC

Detection techniques	Number of samples	Ss (%)	Ov (%)	Al (%)	Tsp (%)	Hw (%)	Tt (%)	Hn (%)	Dc (%)	Total (%)
FECT	646	15 (2.32)	6 (0.93)	4 (0.62)	4 (0.62)	3 (0.46)	2 (0.31)	2 (0.31)	1 (0.15)	37 (5.73)
APC	646	34 (5.26)	0	0	0	0	0	0	0	34 (5.26)

Molecular study of S. stercoralis with PCR

A total of 34 samples exhibiting positive results for *S. stercoralis* were detected using the agar plate culture technique. These samples were collected from five provinces located within the lower northern region of Thailand. Specifically, 12 of the positive samples originated from Phitsanulok, 11 from Sukhothai, 4 from Phetchabun, 4 from Phichit, and 3 from Utai-Thani.

From every positive sample, only a single filariform larva was chosen for inclusion in this study, a process facilitated through the utilization of Stereo Microscopes. The designated larva was subsequently employed in the subsequent segment for the purpose of DNA extraction, a procedure that had been outlined in a prior publication. Fig. 2 displays a distinct photograph of the filariform larva.

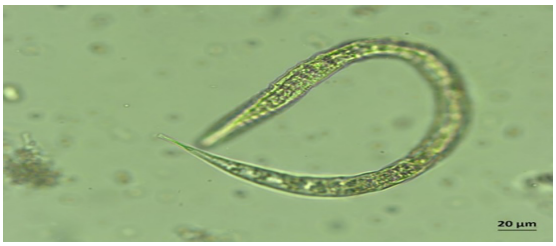

Fig. 2: Filariform larva of *Strongyloides* spp. detected using the Agar plate culture technique and visualized at a magnification of 200 times (Original image)

Visualization of PCR products

After conducting PCR with the 18S primers (MSP4F/StrongR), the products of 586 base pairs were subjected to electrophoresis. Out of the total 34 larvae, successful PCR products were obtained, exhibiting varying degrees of intensity. Specifically, in the Sukhothai samples, 8 strong bands and 3 faint bands were

observed (Fig. 3a). Among the 12 Phitsanulok samples, 10 displayed strong bands while 2 exhibited faint bands (Fig. 3b). All 4 Phetchabun samples revealed strong bands. In the case of the 4 Phichit samples, 2 strong bands and 2 faint bands were noted. Lastly, within the 3 Utai-Thani samples, 2 exhibited strong bands while 1 displayed a faint band, as illustrated in Fig. 3c. In summary, there were a total of 26 strong PCR products and Faint PCR products.

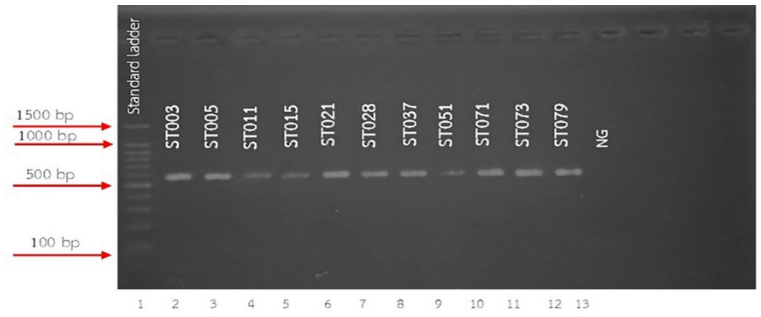

Fig. 3a: PCR products in Sukhothai samples, for details, see text

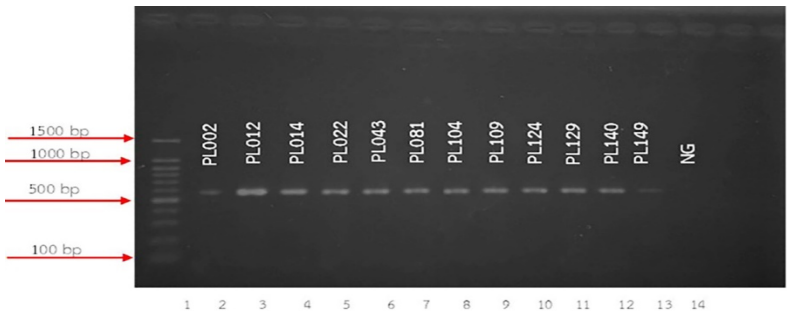

Fig.3b: PCR products in Phitsanulok samples, for details, see text

Fig. 3c: PCR products in Phetchabun samples (Lane 2-5), Phichit samples (Lane 6-9), and Utai-Thani samples (Lane 10-12). For details, see text

DNA sequencing and database searching

Utilizing PCR methodology, we amplified the genomic DNA extracted from thirty-four *Strongyloides* spp. larvae. These larvae were sourced from cultures on agar plates originating from distinct regions, including Phitsanulok (12), Sukhothai (11), Phetchabun (4), Phichit (4), and Utai-Thani (3). Subsequently, we

selected 26 of these PCR products for commercial sequencing. Twenty-six sequences were then subjected to a BLASTN search against the GenBank database at the National Center for Biotechnology Information (NCBI). All searched sequences from the database were *S. stercoralis* gene for 18S ribosomal RNA. Among these, seven sequences displayed significant alignment with the *S. stercoralis* gene, CAR (Accession no. LC085481.1), exhibiting an identity range of 99% to 100%. Additionally, eight sequences exhibited 100% identity with the *S. stercoralis* gene, MMR (Accession no AB923888.1), while eleven sequences exhibited the highest similarity to the *S. stercoralis* gene, KHM (Accession no KU724128.1), with identity percentages ranging from 98% to 100% (Table 4).

Table 4: BLASTN results of *Strongyloides* spp. larvae 18S rDNA sequences in lower northern Thailand

NO.	Code	Maximum identity	BLASTN				
			Accession number	Total score	Query coverage (%)	E value	Identity (%)
1	PL012	*Strongyloides stercoralis*	KU724128.1	1127	100	0.0	99
2.	PL014	*Strongyloides stercoralis*	AB923888.1	1044	100	0.0	100
3	PL022	*Strongyloides stercoralis*	KU724128.1	1127	100	0.0	100
4.	PL043	*Strongyloides stercoralis*	LC085481.1	1127	100	0.0	100
5.	PL081	*Strongyloides stercoralis*	LC085481.1	1127	100	0.0	99
6.	PL104	*Strongyloides stercoralis*	LC085481.1	1127	100	0.0	99
7.	PL109	*Strongyloides stercoralis*	LC085481.1	1127	100	0.0	99
8.	PL124	*Strongyloides stercoralis*	KU724128.1	1094	100	0.0	99
9.	PL129	*Strongyloides stercoralis*	KU724128.1	1094	100	0.0	100
10.	PL140	*Strongyloides stercoralis*	LC085481.1	1127	100	0.0	97
11.	ST003	*Strongyloides stercoralis*	AB923888.1	1044	100	0.0	100
12.	ST005	*Strongyloides stercoralis*	AB923888.1	1044	100	0.0	100
13.	ST021	*Strongyloides stercoralis*	KU724128.1	1094	100	0.0	99
14.	ST028	*Strongyloides stercoralis*	KU724128.1	1094	100	0.0	99
15.	ST037	*Strongyloides stercoralis*	AB923888.1	1044	100	0.0	100
16.	ST071	*Strongyloides stercoralis*	AB923888.1	1044	100	0.0	100
17.	ST073	*Strongyloides stercoralis*	AB923888.1	1044	100	0.0	100
18.	ST079	*Strongyloides stercoralis*	LC085481.1	1127	100	0.0	99
19.	PB034	*Strongyloides stercoralis*	AB923888.1	1044	100	0.0	100
20.	PB038	*Strongyloides stercoralis*	KU724128.1	1094	100	0.0	99
21.	PB063	*Strongyloides stercoralis*	KU724128.1	1094	100	0.0	99
22.	PB107	*Strongyloides stercoralis*	KU724128.1	1094	100	0.0	98
23.	PC044	*Strongyloides stercoralis*	KU724128.1	1094	100	0.0	99
24.	PC079	*Strongyloides stercoralis*	AB923888.1	1044	100	0.0	100
25.	UT012	*Strongyloides stercoralis*	KU724128.1	1094	100	0.0	99
26.	UT051	*Strongyloides stercoralis*	AB923888.1	1044	100	0.0	100

Reconstruction of phylogenetic trees

Twenty-six 18S rDNA sequences (comprising 568 base pairs each) from *Strongyloides* spp. larvae were obtained across five provinces in lower-northern Thailand. These sequences were employed to construct a phylogenetic tree using the distance-based Neighbor-Joining method, as depicted in Fig. 4. The analyzed sequences formed distinct clusters, segregating into three primary clades. The first clade, comprising 14 sequences (ST021, ST073, ST037, ST071, PL129, PL043, PL014,

PL109, PL081, PB038, PB107, PC044, PC079, UT051), exhibited a close association with sequences from Lao (Accession no. KU962180, KU962179), Myanmar (Accession no. AB923888), and Japan (Accession no. AB453316, AB453315). The second clade, composed of 5 sequences (PB034, PB063, PL012, PL104, ST028), appeared to be genetically distinct from any sequences present in the database. Lastly, the third clade, containing 7 sequences (ST005, ST003, ST079, PL022, PL124, PL140, UT012), shared genetic characteristics with sequences from both Lao (Accession no. KU96217981) and Japan (Accession no. AB453314). Not all these 26 sequences of S. stercoralis were submitted to the GenBank accession any. OR454415-OR454440.

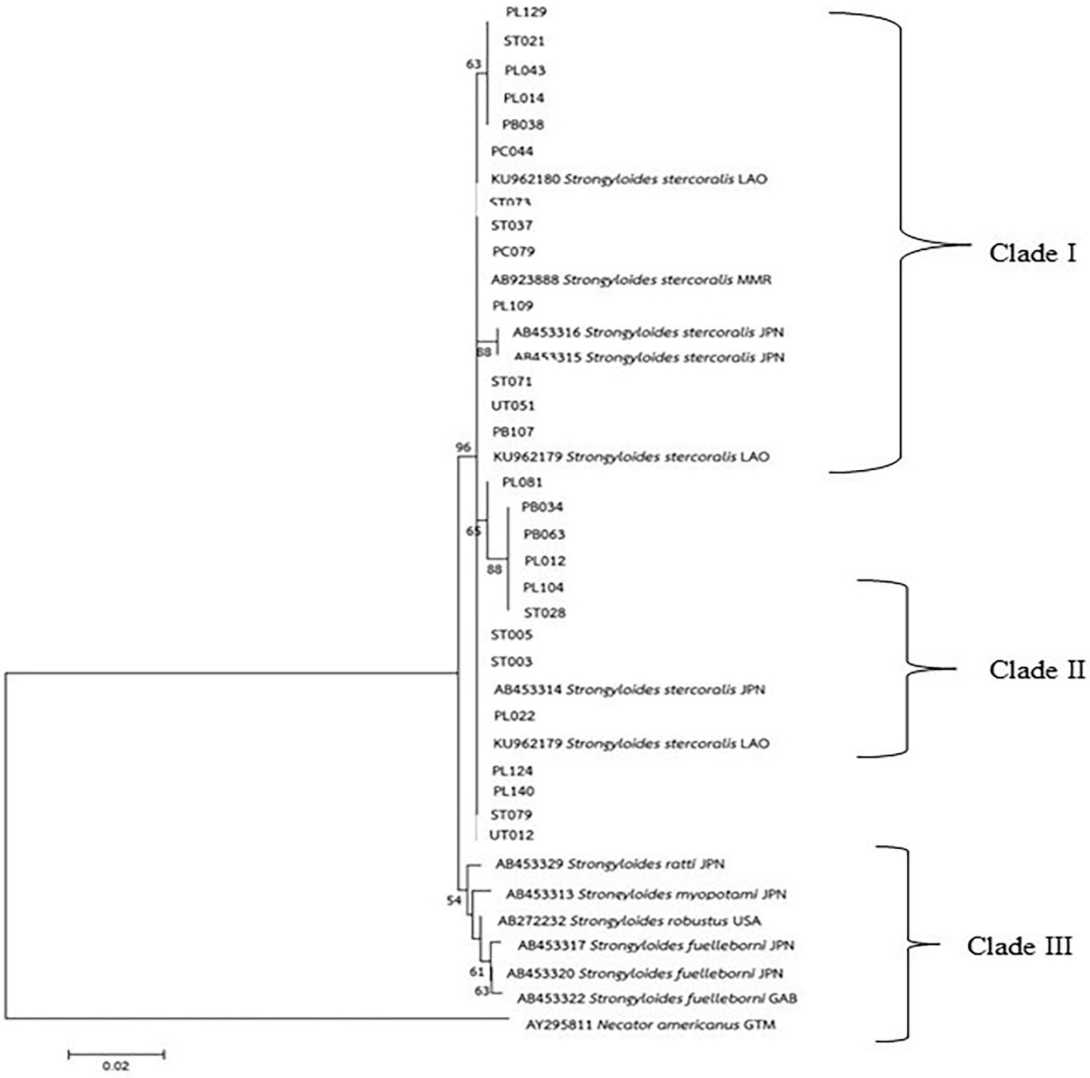

Fig. 4: Neighbor-Joining phylogenetic Tree of *S. stercoralis* 18S rDNA sequences generated using MEGA version 7.0 with kimura 2-parameter model and 1000 bootstrap eplicates

Discussion

The study findings indicated that rubber plantation areas in lower north Thailand exhibited a prevalence rate of helminthic infections was 8.82%, Among 646 fecal samples analyzed, 57 tested positive for parasitic infections. Notably, this rate appears comparatively lower than previous studies on helminthiasis in Thailand, which ranged from 9.79% to 18.1% (11-12). The present investigation scrutinized the prevalence of STH within the rubber plantation domains spanning five provinces in the Thai lower north, detecting 44 positive cases (6.81%). This incidence rate resonates with prior observations regarding STH prevalence across four distinct regions of Thailand: southern (19.4%), northeastern (7.9%), north (6.8%), and middle (4.4%) (11). Among the STH infections, hookworm was the most prevalent at 10.9%, followed by *S. stercoralis* (3.4%) and *T. trichiura* (2.1%) (14). However, in the rubber plantation region of southern Thailand, it is worth noting that the prevalence of *S. stercoralis* has shown declining trend over the years, dropping from 28.5% in 2009 to 8% in 2014, and subsequently to 2.7% in 2017 (14). In our study, the most prevalent parasite was *S. stercoralis* (5.41%). Notably, the current prevalence rates were higher compared to helminth infection rates in north Thailand, where prevalence of helminth infection was 17.7%, with *S. stercoralis* accounting for 2.1% and other helminthic infections like the *O. viverrini* at (10.0%), *Taenia* spp. (0.8%) (11). Our findings indicate a higher prevalence, which could be attributed to the focus of our study on rubber plantation areas a conducive environment for STH, including *S. stercoralis*, to thrive. Additionally, Wisetmora et al. 2024, report prevalence of *Strongyloides* spp. in rodent 92.30% in Northern Thailand (15). A study in Argentina also highlighted zoonotic potential of *S. stercoralis* (16).

The current investigation identified 34 positive samples for *S. stercoralis* through APC out of 646 samples (5.26%) collected from rubber plantation areas across five provinces in the lower north of Thailand. From each sample, only one filariform larva of *S. stercoralis* was carefully selected under stereo microscopes for DNA extraction, destined for PCR analysis and subsequent sequencing targeting the 18S rRNA region using our custom primers, 18S (MSP4F/StrongR), resulting in a PCR product of 568 base pairs (17). This discrepancy may stem from the utilization of genus-specific primers, as opposed to *S. stercoralis* 18S rRNA-specific primers, potentially leading to non-specific amplification of other parasitic genes or indicating multiple types of infection (18). In this study, we focused on analyzing the highly variable region of the 18S ribosomal RNA gene (rDNA) within the species *S. stercoralis*, following a methodology akin to previous research. This approach targets the hypervariable region of the 18S rDNA, known for its diagnostic significance in discerning strains of *S. stercoralis* within the broader context of *Strongyloides* spp. (19). A phylogenetic tree was constructed using nucleotide sequences obtained from the PCR products of all 26 positive samples, representing 70.59% of the total 34 positive samples. This analysis confirmed that all 26 samples collected from various provinces were identified as *S. stercoralis*, closely aligning with strains found in Lao (Accession nos. KU962180, KU962179, KU96217981), Myanmar (Accession no. AB923888), and Japan (Accession nos. AB453316, AB453315, AB453314). Notably, a study on the diversity of *S. stercoralis* transmitted via soil in the Republic of Lao reported strains closely related to those in Myanmar (Accession no. AB923888) and Japan (Accession nos. AB453316, AB453315, AB453314). This phylogenetic analysis unveils the genetic diversity within the *Strongyloides* genus in the SEA region. Moreover, it underscores the high prevalence of *S. stercoralis* in numerous

countries such as Cambodia, the Republic of Lao, and Thailand (20).

Conclusion

Our study highlights a prevalence rate of 8.82% for all helminthic infections with *S. stercoralis* as the predominant parasite, accounting for 5.41%. Sequencing of the PCR products identified *S. stercoralis* strains closely related to those reported in the Republic of Lao, Myanmar, and Japan, suggesting genetic diversity within the species. These findings emphasize the imperative of implementing comprehensive public health strategies for surveillance, treatment, and prevention of parasite transmission.

Acknowledgements

This study was supported by Naresuan University (NU), and National Science, Research and Innovation Fund (NSRF), Thailand (Grant No. R2566B058). We would like to thank Professor Jorge Aigla, M.D and Mr.Olalekan Israel Aiikulola, visiting professors to the Faculty of Medical Science, Naresuan University for their assistance in editing this manuscript.

Conflicts of interest

We declare that we have no conflict of interest.

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