Research Article

Molecular Detection and Phylogenetic Analysis of Tick-Borne Pathogens in Goats in Kanchanaburi, Khon Kaen, and Chaiyaphum, Thailand

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Abstract

Tick-borne pathogens (TBPs) are a major health concern for small ruminants, leading to economic losses through reduced productivity and increased mortality. This study aimed to investigate the presence and genetic characteristics of TBPs in goats from three provinces in Thailand: Kanchanaburi, Khon Kaen, and Chaiyaphum, using PCR-based molecular methods. A total of 258 goat blood samples were tested for *Theileria* spp., *Theileria ovis, Theileria orientalis, Babesia ovis, Anaplasma ovis,* and *Anaplasma marginale.* Only *Theileria* spp. (8/258; 3.10%) were detected, while the other pathogens were not found. Positive samples were sequenced, and phylogenetic analysis of 18S rRNA gene fragments revealed that the isolates clustered with *Theileria luwenshuni* previously reported from Thailand, China, and Myanmar. These findings confirm the presence of *T. luwenshuni* in goats in these regions and provide insights into its genetic relatedness to regional strains. Continued surveillance and molecular monitoring are recommended to better understand the epidemiology of TBPs in Thailand's goat population.

Keywords: Tick-borne pathogens, *Theileria luwenshuni*, Goats, Molecular detection, Phylogenetic analysis, Thailand

การตรวจวิเคราะห์ทางโมเลกุลและการวิเคราะห์ความสัมพันธ์เชิงวิวัฒนาการของเชื้อก่อโรคที่นำโดยเห็บจากตัวอย่างเลือดแพะในจังหวัดกาญจนบุรี ขอนแก่น และชัยภูมิ ประเทศไทย

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บทคัดย่อ

เชื้อก่อโรคจากเห็บ (Tick-borne pathogens: TBPs) เป็นปัญหาสำคัญที่ส่งผลกระทบต่อสุขภาพของสัตว์เคี้ยวเอื้องขนาดเล็ก และก่อให้เกิดความเสียหายทางเศรษฐกิจจากผลผลิตที่ลดลงและอัตราการตายที่เพิ่มขึ้น งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาการติดเชื้อและความหลากหลายทางพันธุกรรมของ TBPs ในแพะจาก 3 จังหวัดของประเทศไทย ได้แก่ กาญจนบุรี ขอนแก่น และชัยภูมิ โดยใช้เทคนิค PCR ตรวจหาเชื้อ *Theileria* spp., *Theileria ovis, Theileria orientalis, Babesia ovis, Anaplasma ovis* และ *Anaplasma marginale* ในตัวอย่างเลือดแพะจำนวน 258 ตัวอย่าง พบเฉพาะเชื้อ *Theileria* spp. (8/258; ร้อยละ 3.10) เท่านั้นที่ให้ผลบวก โดยเชื้อที่ตรวจพบได้ถูกนำมาหาลำดับเบส และผลการวิเคราะห์ความสัมพันธ์เชิงวิวัฒนาการบริเวณยีน 18S rRNA และพบว่าเชื้อจัดอยู่ในกลุ่มเดียวกับ *Theileria luwenshuni* ที่เคยมีรายงานจากประเทศไทย จีน และเมียนมา ผลการศึกษานี้แสดงให้เห็นถึงการมีอยู่ของ *T. luwenshuni* ในแพะในพื้นที่ศึกษา และแสดงให้เห็นถึงความเกี่ยวข้องทางพันธุกรรมกับสายพันธุ์ในภูมิภาค จึงควรมีการเฝ้าระวังและติดตามทางโมเลกุลอย่างต่อเนื่อง เพื่อเข้าใจระบาดวิทยาของเชื้อ TBPs ในประชากรแพะของประเทศไทยให้ดียิ่งขึ้น

คำสำคัญ : เชื้อก่อโรคที่นำโดยเห็บ, *Theileria luwenshuni*, แพะ, การตรวจวินิจฉัยระดับโมเลกุล, การวิเคราะห์ความสัมพันธ์เชิงวิวัฒนาการ, ประเทศไทย

Introduction

Tick-borne pathogens (TBPs) cause diseases in livestock that result in substantial economic losses due to reduced productivity, increased mortality, and the cost of veterinary care (Alessandra and Santo 2012). These pathogens include protozoa (*Babesia* spp. and *Theileria* spp.) and bacteria (*Anaplasma* spp., *Ehrlichia* spp., and *Rickettsia* spp.).

Babesia ovis and *Babesia motasi* are the main species of the genus *Babesia* responsible for babesiosis in sheep and goats. *B. ovis* is highly pathogenic, resulting in acute hemolytic anemia, fever, hemoglobinuria, jaundice, and mortality. *B. motasi*, although generally less pathogenic than *B. ovis*, can still cause notable morbidity and production losses, particularly under conditions of stress or immunosuppression (Ijaz et al., 2013). Theileriosis, caused by various species of *Theileria*, is a significant protozoan disease affecting sheep and goats, particularly in tropical and subtropical regions. *Theileria lestoquardi, Theileria uilenbergi,* and *Theileria luwenshuni* are highly pathogenic and associated with severe clinical signs (Islam et al., 2021). In contrast, *Theileria recondite*, *Theileria separate*, and *Theileria ovis*, generally cause benign theileriosis (Yin et al., 2004). However, they can still lead to production losses under stressful conditions or in immunocompromised animals.

Among bacterial pathogens, *Anaplasma phagocytophilum* and *Anaplasma ovis* are significant contributors to tick-borne diseases in small ruminants (Yousefi et al., 2017). *A. ovis* is primarily responsible for ovine anaplasmosis, a disease characterized by fever, progressive anemia, jaundice, weight loss, and reduced productivity. The infections of *A. ovis* are often asymptomatic and can be more severe in goats than in sheep, particularly in stressed or weak animals (Mason et al., 2017).

Goats are an important part of Thailand's agricultural economy, especially in rural areas where they expose to many vector-borne diseases transmitted by arthropods, including ticks and hematophagous insects. The occurrence of TBPs has been investigated in various regions of Thailand. Although earlier studies focused largely on cattle and buffalo (Altangerel et al., 2011; Jirapattharsate et al., 2016; Jirapattharsate et al., 2017), recent reports have also documented TBPs in goats, including molecular detection of *Theileria*, *Babesia*, and *Anaplasma* species in Thailand (Aung et al., 2022; Udonsom et al., 2022; Aung et al., 2024) However, data remain limited in several parts of the country, particularly in central and lower northeastern Thailand, warranting further investigation. This study aimed to investigate the presence of tick-borne pathogens in goats from three provinces in Thailand-Kanchanaburi, Khon Kaen, and Chaiyaphum-using PCR-based molecular detection methods. These provinces were selected due to their significance as major goat-rearing regions, their documented exposure to tick infestations, and their representation of different agro-ecological zones across central, northeastern, and western Thailand. The targeted pathogens included *Theileria* spp., *T. orientalis, T. ovis, B. ovis, A. ovis,* and *A. marginale.* In addition to assessing the prevalence of these pathogens, we conducted phylogenetic analysis to evaluate the genetic diversity of the detected species and their relationships to previously reported strains.

Materials and methods

1. Ethical statement

Residual blood samples, originally collected for the diagnostic investigation of toxoplasmosis and neosporosis in cattle and goats, were utilized in the present study. Ethical approval for the use of goat blood samples was granted by

the Ethics and Animal Care and Use Committee, Faculty of Veterinary Science, Mahidol University (Permit No. MUVS-2023-05-35).

2. Animal sample and DNA extraction

A total of 258 whole-blood samples were collected from goats randomly selected between April and May 2021, regardless of breed, age, or sex. Sampling was conducted from both backyard and farm settings in three provinces-Kanchanaburi (n = 114), Khon Kaen (n = 60), and Chaiyaphum (n = 84)-selected based on accessibility and their relevance to goat farming (Figure 1). Approximately 2.5 mL of blood was obtained from each animal via jugular venipuncture using sterile 5 mL EDTA vacuum tubes and stored at a cool temperature until laboratory processing. The sample size was determined by the availability of residual blood samples originally collected for diagnostic purposes and was not statistically calculated.

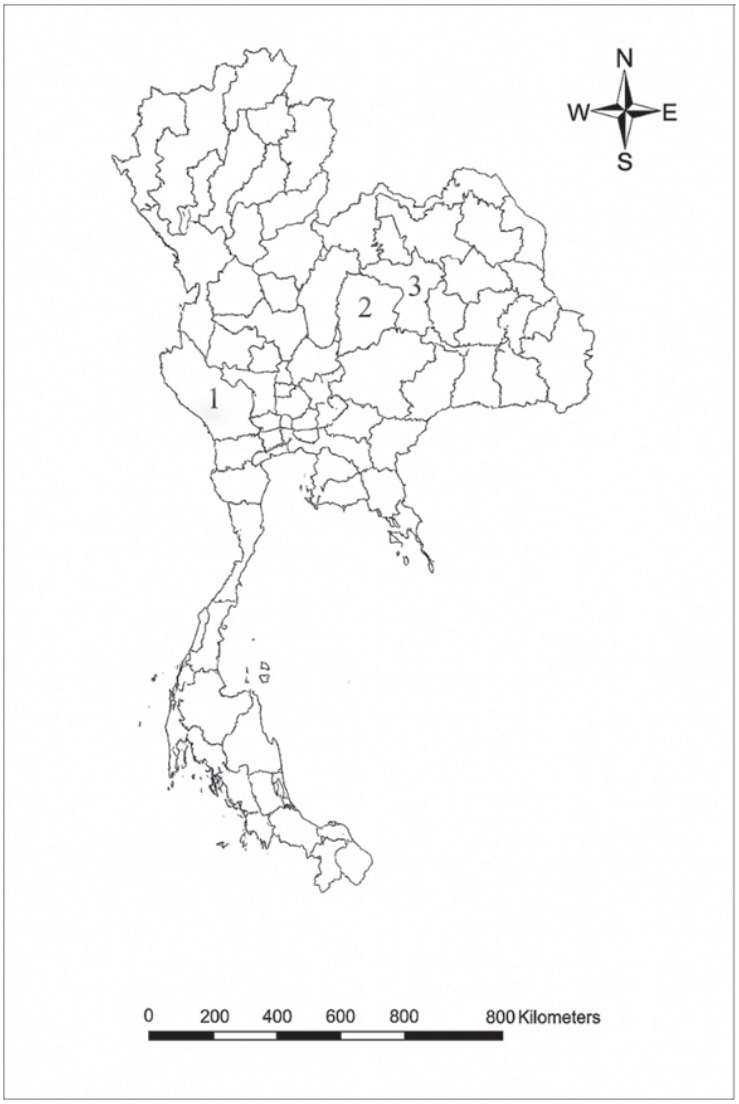


Figure 1. The sampling areas of goats in Thailand were located in three provinces: (1) Kanchanaburi, (2) Chaiyaphum, and (3) Khon Kaen.

3. Genomic DNA extraction

Genomic DNA was extracted from approximately 200 μL of whole blood using the G-spin™ Total DNA Extraction Kit (iNtRON Biotechnology, Inc., Seongnam, South Korea), in accordance with the manufacturer's instructions. The resulting DNA was stored at-20 °C until further analysis. The purity and concentration of the extracted DNA were evaluated using a NanoDrop™ 2000 spectro-photometer (Thermo Fisher Scientific, Waltham, MA, USA) prior to downstream applications.

4. Detection of TBPs and DNA sequencing

Molecular detection was performed using primers specific to the target genes of *Theileria* spp., *T. orientalis*, *T. ovis*, *B. ovis*, *Anaplasma ovis*, and *A. marginale*. The selected primer sets target well-conserved genes and has been previously validated in goats and other ruminants in both international and Thai field studies. Their use in this study was based on prior successful applications demonstrating high sensitivity and specificity under similar field conditions (Aktaş et al., 2005; Altay et al., 2005; Torina et al., 2012; Jirapattharasate et al., 2017; Udonsom et al., 2022). The sequences of all primers employed, along with the corresponding PCR conditions, are detailed in Table 1.

Each PCR reaction was prepared in a 25 μL total volume, containing 5 μL of 5x OneTaq Standard Reaction Buffer, 0.5 μL of dNTPs, 0.2 μM of forward and reverse primers, and 0.125 μL of OneTaq DNA polymerase (New England Biolabs, Ipswich, MA, USA). The remaining volume was adjusted with nuclease-free distilled water. Positive control DNA samples previously confirmed for *Theileria* spp. and *A. ovis* (Udonsom et al., 2022), as well as *A. marginale* (Jirapattharasate et al., 2017), were included in the PCR assays. Nuclease-free water was used as a negative control. Due to the unavailability of positive control DNA for *Babesia ovis* and *Theileria ovis*, verification of amplicon sizes was performed through sequencing. PCR products were electrophoresed on 1.5% agarose gels using 1x TAE buffer and visualized under UV light following staining with FluoroDye™ DNA Fluorescent Loading Dye (SMOBIO Technology, Hsinchu City, Taiwan). Bands corresponding to positive amplicons were excised and purified using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. DNA concentrations were assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The purified products were submitted for Sanger sequencing at Macrogen (Seoul, Korea). Resulting nucleotide sequences were processed and aligned using BioEdit version 7.2.6 (Tom Hall, Ibis Biosciences, CA, USA), and their identities were confirmed via BLASTn searches against the GenBank database. Pairwise sequence comparisons and percent identity calculations were conducted using MEGA 11 software (Tamura et al., 2021).

5. Phylogenetic Analysis

The nucleotide sequences generated in this study were compared with reference sequences available in public genetic databases using MEGA version 11 software (Tamura et al., 2021). Multiple sequence alignments for each genetic locus were conducted using the MUSCLE algorithm, and phylogenetic relationships were inferred employing either the neighbor-joining or maximum likelihood method. The robustness of the resulting phylogenetic trees was assessed through bootstrap analysis based on 1,000 replicates.

Table 1. List of target genes and primers used for PCR assays.

Target gene	Assay	Primer Sequence (5'-> 3')		Fragment (bp)	Annealing Temp. (°C)	Reference
		Forward	Reverse			
A. ovis (MSP4)	PCR	TGAAGGGAGCGG GGTCATGGG	GAGTAATTGCAGC CAGGCACTCT	347	62	(Torina et al., 2012)
A. marginale (MSP4)	PCR	CTGAAGGGGGA GTAATGGG	GGTAATAGCTGCC AGAGATTCC	344	60	(Torina et al., 2012)
B. ovis (18S RNA)	PCR	TGGGCAGGACC TTGGTTCTTCT	CCGCGTAGCGCC GGCTAAATA	549	62	(Aktaş et al., 2005)
T. ovis (18S rRNA)	PCR	TCGAGACCTTC GGGT	TCCGGACATTG TAAAACAAA	520	60	(Altay et al., 2005)
T. orientalis (MPSP)	PCR	CTTTGCCTAGGA TACTTCCT	ACGGCAAGTGG TGAGAACT	776	58	(Ota et al., 2009)
Theileria spp. (18S rRNA)	PCR	GAAACGGCTAC CACATCT	AGTTTCCCCGTG TTGAGT	778	55	(Cao et al., 2013)
	nPCR	TTAAACCTCTTC CAGAGT	TCAGCCTTGCGA CCATAC	581	55	

Abbreviation: Major surface protein 4 gene (MSP4), 18S ribosomal RNA (18S RNA), Major piroplasm surface protein (MPSP), Polymerase chain reaction (PCR), Nested polymerase chain reaction (nPCR).

Results

1. PCR detection of TBPs

In the present study, none of the 258 blood samples tested positive for *B. ovis*, *T. ovis*, *T. orientalis*, *A. ovis*, and *A. marginale*. Only *Theileria* spp. was detected, with an overall infection rate of 3.10% (8/258; 95% CI: 1.35-6.00) among the goat populations. The province-specific prevalence rates were 3.51% in Kanchanaburi (4/114; 95% CI: 0.97-8.74), 1.67% in Khon Kaen (1/60; 95% CI: 0.04-8.95), and 3.57% in Chaiyaphum (3/84; 95% CI: 0.74-10.04).

Table 2. Number of PCR positive samples and infection rate.

	Kanchanaburi (n=114)	Khon Kaen (n=60)	Chaiyaphum (n=84)
A. ovis	0	0	0
A. marginale	0	0	0
B. ovis	0	0	0
Theileria spp.	3.50% (95% CI: 0.97-8.74)	1.67% (95% CI: 0.04-8.95)	3.57% (95% CI: 0.74-10.04)

2. Comparative sequence and Phylogenetic analysis

BLASTn analysis of the partial 18S rRNA gene sequences of *Theileria* spp. obtained in this study (accession numbers: PQ774181-PQ774187) revealed 100% nucleotide identity with *T. luwenshuni* sequences previously isolated from goats in Thailand (OM802538, MZ734312, and MW307320), sheep in China (KC414097), and from goats (LC326009) and a dog (LC602484) in Myanmar. Phylogenetic analysis further demonstrated that the partial sequences of *T. luwenshuni* identified in this study clustered within a single clade alongside *T. luwenshuni* isolates from Thailand, Myanmar, and China (Figure 2).

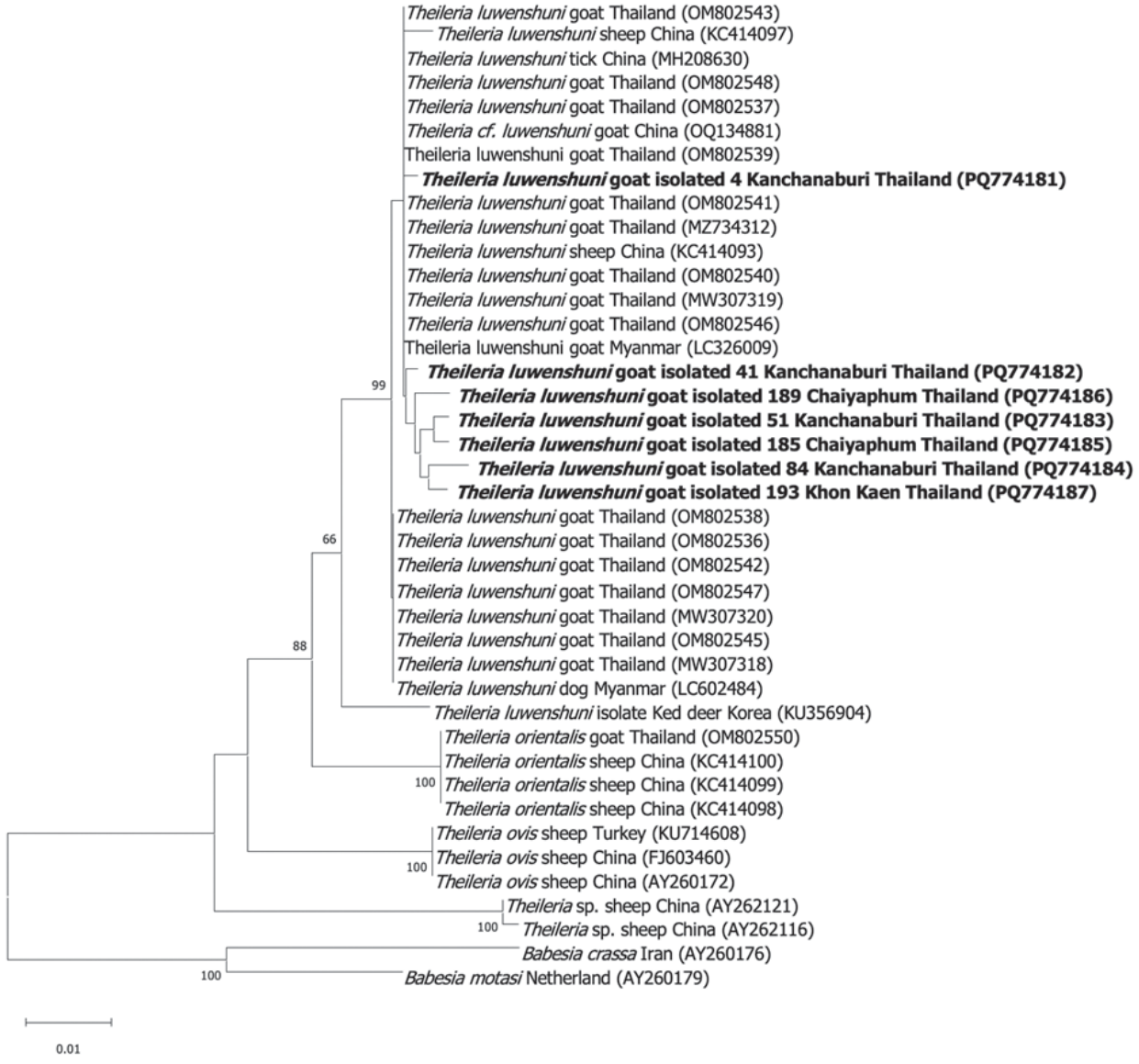


Figure 2. A phylogenetic tree was reconstructed using partial 18S rRNA gene sequences of *T. luwenshuni* identified in this study (shown in bold), alongside representative sequences of other *Theileria* species. The analysis employed the maximum likelihood method with the Kimura 2-parameter model, and the robustness of the tree topology was evaluated through 1,000 bootstrap replications. Bootstrap support values are indicated at each node, with only values exceeding 60 presented. Partial sequences of *Babesia crassa* and *Babaesia motasi* were included as outgroup references to root the tree.

Discussion

Tick-borne pathogens (TBPs) infections pose a significant concern for small-ruminant farming, particularly in tropical and subtropical regions where environmental conditions support the proliferation of arthropod vectors. In Southeast Asia, research on tick-borne diseases has been conducted in countries such as Thailand, Myanmar, Cambodia (Yean et al., 2024), and others including China (Han et al., 2024) and Pakistan (Mushtaq et al., 2021). While recent studies have enhanced the understanding of TBPs in Thailand, including investigations in goats and cattle across various regions, data remain limited for small ruminants in certain areas. Our study contributes to this field by providing new insights into the prevalence and genetic characteristics of TBPs in goats from central and lower northeastern provinces, thereby expanding the existing epidemiological knowledge base.

Among these pathogens, theileriosis-caused by *Theileria* species-is a significant tick-borne disease affecting goats worldwide, with particularly severe impacts in tropical and subtropical climates. The economic consequences of theileriosis can be substantial due to its association with high morbidity and mortality. In Thailand, previous studies have reported varying prevalence rates of *Theileria* spp. in goats depending on geographic location. The prevalence of *Theileria* spp. in goats has been reported to vary across different regions of Thailand, with studies indicating rates of 10.3% in the southern provinces (Udonsom et al., 2022) and up to 41.33% in Watthana Nakhon district, Sa Kaeo province (Kaewhom and Thitasarn 2017). Similar variability is seen across Asia, with reported prevalence rates of 34.7-52.5% in China (Cao et al., 2013), 33.3% in Myanmar (Bawm et al., 2018), 15.4% in Pakistan (Mushtaq et al., 2021), and 8.5% in Bangladesh (Islam et al., 2021).

Molecular identification and phylogenetic characterization of *Theileria* species commonly involve PCR amplification of the 18S rRNA gene, a widely accepted target for species-level discrimination (Yan et al., 2021). The detection of *T. luwenshuni* in goats from all three provinces is consistent with the known presence of ixodid ticks in these regions. Previous surveys have reported the occurrence of *Haemaphysalis* and *Rhipicephalus* spp. in both central and northeastern Thailand, including Kanchanaburi, Khon Kaen, and Chaiyaphum provinces (Altangerel et al., 2011; Jirapattharasate et al., 2016). These tick genera are recognized vectors of *Theileria* species, particularly *T. luwenshuni*. The environmental conditions in these provinces characterized by mixed farming systems, suitable vegetation cover, and seasonal rainfall favor tick survival and transmission. However, detailed entomological data specific to goat farms in the study areas remain limited, highlighting the need for integrated surveillance of both pathogens and vectors to better assess transmission risk. Sequence analysis revealed 99.3-100% similarity to *T. luwenshuni* strains previously reported in southern Thailand, China, and Myanmar. Earlier studies have shown varying prevalence of *T. luwenshuni*, with rates as high as 80% in Yanji Province and 32% in Jinchang Province of China (Cao et al., 2013), and 10.3% in goats from southern Thailand (Udonsom et al., 2022). The close genetic similarity observed between isolates from Thailand and China may be influenced by factors such as shared tick vectors and regional animal movement patterns. This hypothesis is supported by findings from other regions; for instance, *T. ovis* isolates from Saudi Arabia closely resemble those from Turkey (Metwally et al., 2021), suggesting that geographically distant yet genetically similar strains can occur.

In the present study, *A. marginale*, *A. ovis*, and *B. ovis* were not detected in any of the goat blood samples collected from the study areas. While this absence may suggest a low prevalence or absence of these pathogens in the sampled populations, definitive conclusions cannot be drawn without broader epidemiological coverage. Previous studies have reported prevalence rates of *Anaplasma* spp. in goats ranging from 2.3% to 28.7%, depending on the geographic region, host population, and detection methodology employed (Torina et al., 2012; Yousefi et al., 2017). Further investigations incorporating larger, seasonally stratified sample sets and multi-gene detection approaches are warranted to comprehensively assess the prevalence of these tick-borne pathogens in goats across Thailand. A key limitation of this study is the absence of positive control DNA for *B. ovis* and *T. ovis*. Although amplicon sequencing was used for confirmation, the absence of positive amplification standards may have compromised the detection threshold for these targets. This limitation should be considered when interpreting the negative findings and highlights the need for future studies to incorporate validated positive controls for all targeted pathogens.

Conclusions

This study provides new molecular evidence of *T. luwenshuni* infection in goats from Kanchanaburi, Khon Kaen, and Chaiyaphum provinces, expanding the known geographic distribution of this pathogen in Thailand. Phylogenetic analysis revealed close genetic relationships between the Thai isolates and those from China and Myanmar, suggesting possible transboundary transmission routes. These findings underscore the importance of continued surveillance and tick control programs, particularly in regions with expanding goat production. Although *T. luwenshuni* has been previously reported, our data contribute new regional insights relevant to small-ruminant health and veterinary epidemiology in Thailand.

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