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Original Article

Molecular Detection of *Theileria* and *Babesia* in a Diversity of Stomoxyini Flies (Diptera: Muscidae) from Khao Yai National Park, Thailand

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Abstract

Blood protozoan species of the genera *Theileria* and *Babesia* are known to infect both domestic and wild animals. This study was carried out to detect *Theileria* and *Babesia* DNA in Stomoxyini flies collected in Khao Yai National Park, Thailand. A total of 110 Stomoxyini flies including 6 species, *Stomoxys calcitrans*, *Stomoxys indicus*, *Stomoxys pullus*, *Stomoxys uruma*, *Haematobosca sanguinolenta* and *Haematostoma austeni*, were examined by amplification against the 18S rRNA gene of the *Theileria/Babesia* species and then the PCR products were sequenced for species identification by comparison with published sequences from the GenBank database. Ten (9.1%) out of 110 samples were positive against the 18S rRNA gene of the *Theileria/Babesia* species. For sequence analysis, 7 samples (6.4%) were identified as *Theileria* sp. and showed high identity (99%) with *Theileria* sp. (JQ751279) and *T. cervi* (HQ184406 and HQ184411), while 3 samples (2.7%) were identified as *Babesia canis vogeli* and showed 100% identity with reported sequences of *B. canis vogeli* (AB083374 and HM590440). Phylogenetic relationships among the sequenced samples showed that the *Theileria* sp. appeared in the same group as *T. cervi*, while *B. canis vogeli* appeared in a distinct group. This study is the first report on a variety of Stomoxyini flies infected with the *Theileria* and *Babesia* species in Thailand conducted using molecular identification techniques.

Keywords: *Babesia*, diversity, ribosomal RNA, Stomoxyini flies, *Theileria*

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Introduction

Stomoxyini flies (Diptera: Muscidae) are obligate haematophagous insects of considerable medical and veterinary significance (Zumpt, 1973; Baldacchino et al., 2013). They are classified into the tribe Stomoxyini in the subfamily Muscinae, and more than 50 species in 10 genera have been recorded (Crosskey, 1993). Nine species from 5 genera have been described in Thailand: *Stygeromyia* (1 species), *Stomoxys* (5 species), *Haematobosca* (1 species), *Haematostoma* (1 species) and *Haematobia* (1 species) (Tumrasvin and Shinonaga, 1978; Changbunjong et al., 2012). The cosmopolitan species, *Stomoxys calcitrans*, can attack most large mammals including domestic cattle, horses, donkeys, dogs, swine, sheep, goats, camels and wild animals of the families Bovidae, Cervidae, Equidae, Canidae and Felidae (Moon, 2009). They are mechanical vectors of several viral, bacterial, protozoan and helminth pathogens but can also be a biological vector of different nematodes (Zumpt, 1973; Baldacchino et al., 2013).

Theileriosis and Babesiosis are important protozoan diseases in wild and domestic animals, and both are mainly transmitted by hard ticks of the family Ixodidae (Jongejan and Uilenberg, 2004). These diseases have a major impact on livestock production in tropical and subtropical regions (Uilenberg, 1995; Altay et al., 2008; Perera et al., 2014). In wild animals, although these diseases are typically asymptomatic, they can be pathogenic under certain circumstances such as under stress, immunosuppression or malnutrition (Penzhorn, 2006; Silveira et al., 2011; Zanet et al., 2014). Several methods have been developed to determine the infection rates of blood protozoa within vector populations. For instance, *Theileria* and *Babesia* merozoites can be detected within the midgut of infected *S. calcitrans* by Giemsa staining (Hadi and Al-Amery, 2012). Although this method is relatively easy to perform, it is not possible to differentiate between merozoites belonging to different *Theileria* and *Babesia* species. Molecular diagnostic assays such as polymerase chain reaction (PCR) have been developed and used as epidemiological and diagnostic tools for the detection and identification of *Theileria* and *Babesia* in both animals and arthropod vectors (Fyumagwa et al., 2011; Githaka et al., 2012; Razmi et al., 2013). Various target genes have been used as genetic markers to identify these parasites. The 18S ribosomal RNA (18S rRNA) gene, consisting of both conserved and variable regions, makes a suitable marker for detection and genetic characterization of blood parasites' DNA (Chansiri et al., 1999; Githaka et al., 2012).

Haematophagous vectors contain infected host blood and the pathogen; the analysis of these vectors is a reliable tool to reveal the existence of pathogens in the specific area (Chansiri et al., 1999; Fyumagwa et al., 2011; Githaka et al., 2012; Razmi et al., 2013; Sumrandee et al., 2015). However, little knowledge is available of blood protozoa in Stomoxyini flies. Thus, this study was carried out to detect blood protozoa belonging to *Theileria* spp. and *Babesia* spp. in the Stomoxyini flies from Khao Yai National Park, Thailand and to determine their phylogenetic relationship with other related species based on the 18S rRNA gene sequences.

Materials and Methods

Fly collection: The study was conducted from February to June 2012 in Khao Yai National Park, Nakhon Ratchasima Province, Thailand: (1) N14°25´43.0´´, E101°22´59.1´´; (2) N14°25´37.2´´, E101°23´12.1´´; (3) N14°24´55.1´´, E101°22´33.4´´; (4) N14°24´54.74´´, E101°22´14.15´´; (5) N14°25´10.83´´, E101°22´12.2´´ (Figure 1). This area includes evergreen forests and grasslands. More than 2,000 species of plants, 320 species of birds and 70 species of mammals are found in the area. Stomoxyini flies were collected using Vavoua traps. All specimens were identified morphologically under a stereomicroscope using taxonomic keys (Zumpt, 1973; Tumrasvin and Shinonaga, 1978). The specimens were collected directly into 1.5 ml microcentrifuge tubes containing 95% ethanol and stored at -20°C for further analysis.

Figure 1 Map of Stomoxyini fly collection sites in Khao Yai National Park

DNA extraction and 18S rRNA amplification: Genomic DNA was extracted from an individual specimen using a QIAamp DNA minikit (QIAGEN, Germany) according to the manufacturer's protocol. Amplification of a 619 basepair (bp) fragment of the 18S rRNA gene was performed using PCR. The primer pair Ba/ThF 5' CCAATCCTGACACAGGGAGGTAGT GACA 3' was the forward primer and Ba/ThR 5'CCCCAGAACCCAAAGACTTTGATTTCTCTCAA G 3' was the reverse primer (Kledmanee et al., 2009). The PCR was performed in a thermocycler (BioRad) in a total reaction volume of 25 µl containing 12.5 µl of QIAGEN multiplex PCR Kit (QIAGEN, Germany), 10.3 µl of nuclease free water, 0.1 µl of 100 µM of forward primer, 0.1 µl of 100 µM of reverse primer and 2 µl of DNA template. The PCR reaction consisted of initial denaturation at 95°C for 15 min, 35 cycles of 94°C for 45 sec, annealing at 70°C for 45 sec, extension at 72°C for 90 sec, and final extension at 72°C for 10 min, followed by indefinite hold at 15°C. Amplified PCR products were separated in 2% agarose gel electrophoresis, and the GeneRulerTM 100 bp DNA ladder (Fermentas, Lithuania) was used as a size marker to visualize the amount and size of DNA fragments present in the sample.

DNA sequencing and phylogenetic analysis: DNA sequencing of PCR products was performed by Bio Basic Canada Inc. (Ontario, Canada). DNA sequences were analyzed using an ABI 3730 XL sequencer and fluorescent dye-terminator sequencing. DNA sequences were performed using Ba/ThF and Ba/ThR primers. The 18S rRNA sequence results were analyzed by several programs. All 18S rRNA sequence results were compared with available sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment of all nucleotide sequences was done using CLUSTAL X (Jeanmougin et al., 1998). Phylogenetic trees were reconstructed using maximum likelihood analysis with bootstrapping (1,000 replications) by MEGA6 software (Tamura et al., 2013). The best nucleotide substitution model was a Kimura 2-parameter with a Gamma distribution parameter (K2+G model). All sequences were compared to published sequences from the GenBank database that originated from other geographic locations globally. *Plasmodium vivax* JQ627157 was included as an outgroup.

Results

A total of 110 Stomoxyini flies, including 55 males and 55 females, were examined and 6 species were identified: *Stomoxys calcitrans*, *Stomoxys indicus*, *Stomoxys pullus*, *Stomoxys uruma*, *Haematobosca sanguinolenta* and *Haematostoma austeni*. Ten (9.1%) out of the 110 samples were positive against the 18S rRNA gene fragment of *Babesia/Theileria* species (Table 1). The positive PCR samples from these flies were sequenced and compared with available sequences. All sequences were deposited in the GenBank databases under accession numbers from KP864649 to KP864658 (Table 2).

Table 1 PCR results for *Theileria* and *Babesia* of Stomoxyini flies collected in Khao Yai National Park

Species	Male	Female	Number	*Theileria* sp. positive	*Babesia canis vogeli* positive
S. calcitrans	15	21	36	1 (2.8%)	1 (2.8%)
S. indicus	0	2	2	1 (50%)	0
S. pullus	10	10	20	1 (5%)	2 (10%)
S. uruma	10	6	16	0	0
H. sanguinolenta	17	13	30	3 (10%)	0
H. austeni	3	3	6	1 (16.7%)	0
Total number	55	55	110	7 (6.4%)	3 (2.7%)

Table 2 GenBank accession numbers of *Theileria* and *Babesia* species in this study

Theileria and *Babesia* species	Stomoxyini fly species	Accession number
Theileria sp. isolate SC 51	*S. calcitrans*	KP864649
Theileria sp. isolate HS 55	*H. sanguinolenta*	KP864650
Theileria sp. isolate HS 57	*H. sanguinolenta*	KP864651
Theileria sp. isolate HS 58	*H. sanguinolenta*	KP864652
Theileria sp. isolate SP 76	*S. pullus*	KP864653
Theileria sp. isolate SI 104	*S. indicus*	KP864654
Theileria sp. isolate HA 106	*H. austeni*	KP864655
Babesia canis vogeli isolate SC 61	*S. calcitrans*	KP864656
Babesia canis vogeli isolate SP 70	*S. pullus*	KP864657
Babesia canis vogeli isolate SP 78	*S. pullus*	KP864658

For sequence analysis, 7 samples (6.4%) from 5 Stomoxyini species (*S. calcitrans*, *S. indicus*, *S. pullus*, *H. sanguiolenta* and *H. austeni*) were identified as *Theileria* sp., while 3 samples (2.7%) from 2 Stomoxyini species (*S. calcitrans* and *S. pullus*) were identified as *B. canis vogeli*. The *Theileria* sequences showed 99% identity with the 18S rRNA gene of the unidentified *Theileria* species (JQ751279) and *T. cervi* (HQ184406 and HQ184411). Of the 3 *Babesia* sequences, all showed 100% identity with *B. canis vogeli* (AB083374 and HM590440). The phylogenetic relationships among the sequenced samples showed that the *Theileria* sp. appeared in the same group as *T. cervi*, while *B. canis vogeli* appeared in a distinct group. These sequences

were clearly separated from other *Theileria* and *Bebesia* species (Figure 2).

Discussion

This is the first report on a variety of the Stomoxyini flies infected with the *Theileria* and *Babesia* species in Thailand using molecular diagnostic assay. Our results suggest that Stomoxyini flies may play an important role as a vector of the parasite. However, the transmission of *Theileria* and *Babesia* by these flies was unknown. Only the report of Hadi and Al-Amery (2011) showed *Theileria* and *Babesia* detected in the midgut of the cosmopolitan species *S. calcitrans* by Giemsa staining. Hence, the presence of specific DNA of these parasites may be derived from host blood meals consumed from infected animals. In several protozoan parasites such as *Trypanosoma* spp. and *Besnoitia besnoiti*, the mechanical transmission through contamination of mouthparts or regurgitation of digestive tract contents by *Stomoxys* species has been demonstrated (Baldacchino et al., 2013).

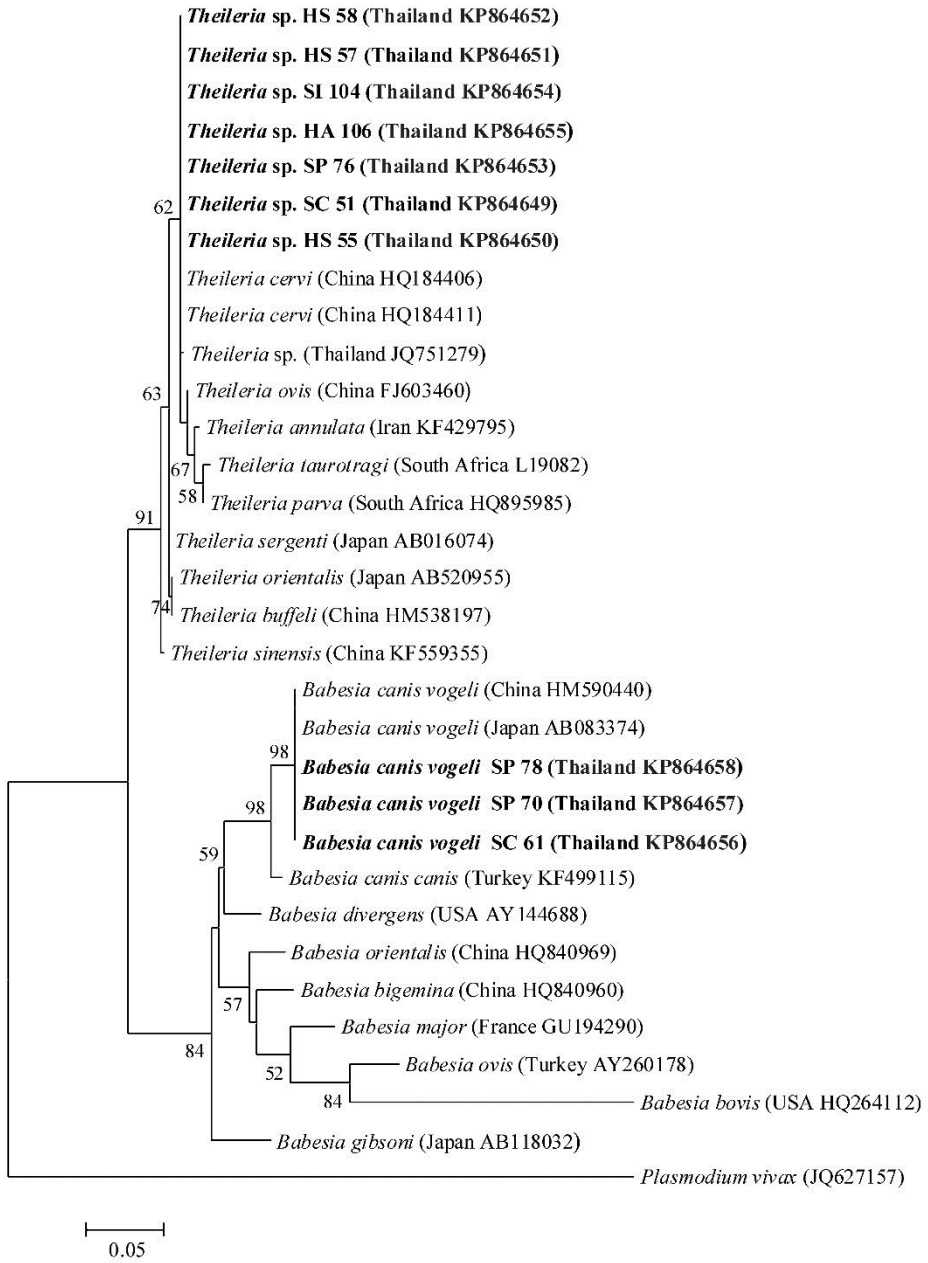

Figure 2 Phylogenetic relationships of *Theileria* and *Babesia* species based on the 18S rRNA gene sequences, which were computed using the maximum likelihood and 1,000 bootstrap replicates

In the present study, 6 species of the Stomoxyini flies, *S. calcitrans*, *S. indicus*, *S. pullus*, *S. uruma*, *H. sanguinolenta* and *H. austeni*, were examined and in almost all species (except *S. uruma*) the parasite was detected. All flies have been reported in different geographical regions of Thailand (Tumrasvin and Shinonaga, 1978; Changbunjong et al., 2012). The large diversity of these flies in Khao Yai National Park is similar to a study conducted by Mihok et al. (1995) in the Nairobi National Park in Kenya; they found 11 species of *Stomoxys* and other genera of

Stomoxyini files including *Prostomoxys*, *Haematobosca*, *Stygeromyia* and *Rhinomusca*.

There have been many reports on both *Babesia* and *Theileria* spp. in domestic and wild animals (Homer et al., 2000; Bishop et al., 2004; Penzhorn, 2006; Yabsley and Shock, 2013). In our study, a molecular survey based on PCR amplification of the 18S rRNA gene was used for the detection and genetic characterization of these parasites. The results of PCR assays were confirmed by DNA sequencing and then the sequences were further analyzed for genotypic identification. These sequences were identified as *Theileria* sp. and *B. canis vogeli*. Both species of the parasites have been reported in Thailand (Buddhachat et al., 2012; Sumrandee et al., 2015). The phylogenetic analysis showed that the *Theileria* sp. detected in this study was closely related to *T. cervi* derived from sika deer (*Cervus nippon*) in China. However, *Theileria* are cosmopolitan parasites that have been detected in wild ruminants (Bishop et al., 2004). There are several deer species from many regions of the world that have been reported to be infected with *T. cervi*, including elk deer (*Cervus canadensis*), white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemonius*), axis deer (*Axis axis*), sika deer (*Cervus nippon*) and reindeer (*Rangifer tarandus*) in the United States (Chae et al., 1999; Garner et al., 2012; Wood et al., 2013); pampas deer (*Ozotoceros bezoarticus*), brown brocket deer (*Mazama gouazoubira*) and marsh deer (*Blastocerus dichotomus*) in Brazil (Silveira et al., 2011; Silveira et al., 2013); and sika deer (*Cervus nippon*) in China and Japan (Inokuma et al., 2004; He et al., 2012). The infection with *T. cervi* in deer is considered benign and clinical symptoms can occur in hosts weakened by other parasites, malnutrition or stress (Chae et al., 1999; Silveira et al., 2011). The principle vector for *T. cervi* in cervids of the US is the lone star tick, *A. americanum* (Wood et al., 2013). In Thailand, Sumrandee et al. (2015) revealed that three tick species (*Haemaphysalis obese*, *H. lagrangei* and *R. microplus*) collected from samba deer (*Cervus unicolor)* in Khao Yai National Park (the same as our study site) were infected with *T. cervi*-like sp. and suggested that sambar deer were a reservoir host of this parasite. Hence, sambar deer seems to be an important host for Stomoxyini flies; we observed many flies (possibly Stomoxyini) aggregated on their bodies. Additionally, Mihok and Clausen (1996) found that the bush buck (*Tragelaphus scriptus*) was the major host for *Stomoxys* spp. in the Nairobi National Park in Kenya.

For *Babesia*, three subspecies of *B. canis* have been reported (Uilenberg et al., 1989): *B. canis rossi*, transmitted by the tick *Haemaphysalis leachi* in South Africa and causing a usually fatal infection in domestic dogs; *B. canis canis*, transmitted by *Dermacentor reticulatus* in Europe and showing a more variable pathogenicity; and *B. canis vogeli*, transmitted by brown dog tick, *Rhipicephalus sanguineus* in tropical and subtropical countries, and leading to a moderate, often clinically unapparent infection (Uilenberg et al., 1989; Costa et al., 2012). In Thailand, *B. canis vogeli* has been found in domestic dogs (Buddhachat et al., 2012; Kongklieng et al., 2015) and also cats (Simking et al., 2010). Based on the sequences of 18S rRNA gene of *B. canis vogeli* detected from the Stomoxyini flies in our study, all these sequences were very similar to *B. canis vogeli* in dogs from China and Japan. We assumed that the flies might obtain the parasite from domestic dogs around the national park. Jenks et al. (2011) detected the domestic dogs roaming as far as 7 km from the park boundary using camera traps. These dogs are most likely to enter the park to hunt prey species. However, *B. canis vogeli* in wildlife species has not been recorded. Other *Babesia* species that have been reported in wild carnivores are such as *B. canis rossi* in side-striped jackal (*Canis adustos*), *B. gibsoni* in fennec fox (*Fennecus dorsalis*), *B. leo* in lion (*Panthera leo*) and *B. microti* in red fox (*Vulpes vulpes*) (Penzhorn, 2006; Fyumagwa et al., 2011; Farkas et al., 2015). Hence, the close contact between domestic dogs and carnivores in the park could also lead to transmission of several infectious diseases such as rabies, canine distemper virus, canine parvovirus and many parasite pathogens.

In conclusion, this is the first report to demonstrate the occurrence of *Theileria* sp. and *Babesia canis vogeli* in Stomoxyini flies from Khao Yai National Park, Thailand. In further studies, the analysis of blood meals should be investigated to determine their main hosts or host preferences and feeding pattern. These may help us to know the effects of Stomoxyini flies on wildlife species.

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บทคัดย่อ

การใช้วิธีทางอณูชีวโมเลกุลตรวจหาเชื้อ *Theileria* และ *Babesia* ในความหลากหลายของแมลงวันคอกสัตว์จากอุทยานแห่งชาติเขาใหญ่ ประเทศไทย

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โปรโตซัวในเลือดสกุล *Theileria* และ *Babesia* สามารถก่อให้เกิดโรคได้ทั้งในสัตว์เลี้ยงและสัตว์ป่า การศึกษานี้ได้ดำเนินการตรวจหาสารพันธุกรรมของเชื้อ *Theileria* และ *Babesia* ในแมลงวันคอกสัตว์ที่เก็บจากอุทยานแห่งชาติเขาใหญ่ในประเทศไทย โดยใช้ตัวอย่างแมลงวันคอกสัตว์จำนวนทั้งสิ้น 110 ตัวอย่างประกอบด้วย 6 ชนิดได้แก่ *Stomoxys calcitrans*, *Stomoxys indicus*, *Stomoxys pullus*, *Stomoxys uruma*, *Haematobosca sanguinolenta* และ *Haematostoma austeni* ตัวอย่างถูกนำไปตรวจหาเชื้อ *Theileria/Babesia* โดยการเพิ่มจำนวนยีนของ 18S ไรโบโซมอล อาร์เอ็นเอ (rRNA) และนำผลผลิตของพีซีอาร์ที่ได้มาหาลำดับเบสเพื่อจำแนกชนิดโดยการเปรียบเทียบกับลำดับเบสจากฐานข้อมูล Genbank การศึกษาพบว่า 10 ตัวอย่าง (ร้อยละ 9.1) ตรวจพบเชื้อ *Theileria/Babesia* การวิเคราะห์ลำดับเบสพบว่า 7 ตัวอย่าง (ร้อยละ 6.4) สามารถระบุชนิดได้เป็น *Theileria* sp. โดยมีความเหมือนกับ *Theileria* sp. (JQ751279) และ *T. cervi* (HQ184406 และ HQ184411) ร้อยละ 99 ขณะที่อีก 3 ตัวอย่าง (ร้อยละ 2.7) สามารถระบุชนิดได้เป็น *Babesia canis vogeli* โดยมีความเหมือนกับ *B. canis vogeli* (HM590440 และ AB083374) ถึงร้อยละ 100 จากความสัมพันธ์เชิงวิวัฒนาการ *Theileria* sp. มีความสัมพันธ์ใกล้ชิดกับ *T. cervi* ขณะที่กลุ่มของ *B. canis vogeli* ถูกจัดอยู่ในกลุ่มเดียวกัน การศึกษานี้เป็นการรายงานครั้งแรกเกี่ยวกับการตรวจพบเชื้อ *Theileria* และ *Babesia* ในความหลากหลายชนิดของแมลงวันคอกสัตว์โดยใช้เทคนิคทางอณูชีวโมเลกุล

คำสำคัญ: *Babesia*, ความหลากหลาย, ไรโบโซมอล อาร์เอ็นเอ, Stomoxyini flies, *Theileria*

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