

# Molecular Detection of Blood Protozoa in Ticks Collected from Cattle in The Buffer Zone of Sai Yok National Park, Thailand

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## Abstract

Ixodid ticks transmit many pathogens to both animals and humans. This study was carried out to detect tick-borne protozoa (*Theileria* and *Babesia*) in ticks collected from cattle in the buffer zone of Sai Yok National Park, Thailand. A total of 30 cattle were examined for tick infestation. Of these, 10 (33.3%) were infested with ticks. A total of 85 ticks were collected and identified as *Rhipicephalus (Boophilus) microplus*. The collected ticks were separated into 50 tick pools by sex and life stage. These pools were examined for *Theileria* and *Babesia* species by polymerase chain reaction (PCR) for amplification of a fragment of the 18S ribosomal RNA (rRNA) gene. Of the 50 tick pools examined, 9 (18%) were infected with the *Theileria* species; of this group, 8 (88.9%) shared 100% identity with the unidentified *Theileria* sp., whereas 1 (11.1%) shared 99% identity with *T. orientalis/sergenti/buffeli*. In the phylogenetic tree, unidentified *Theileria* sp. was closely related to *T. sinensis* and was clearly separated from other *Theileria* species. This study is the first report of *R. microplus* infected with the bovine *Theileria* species in Thailand conducted using molecular identification techniques.

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**Keywords:** *Rhipicephalus microplus*, ribosomal RNA, *Theileria*, tick

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## Introduction

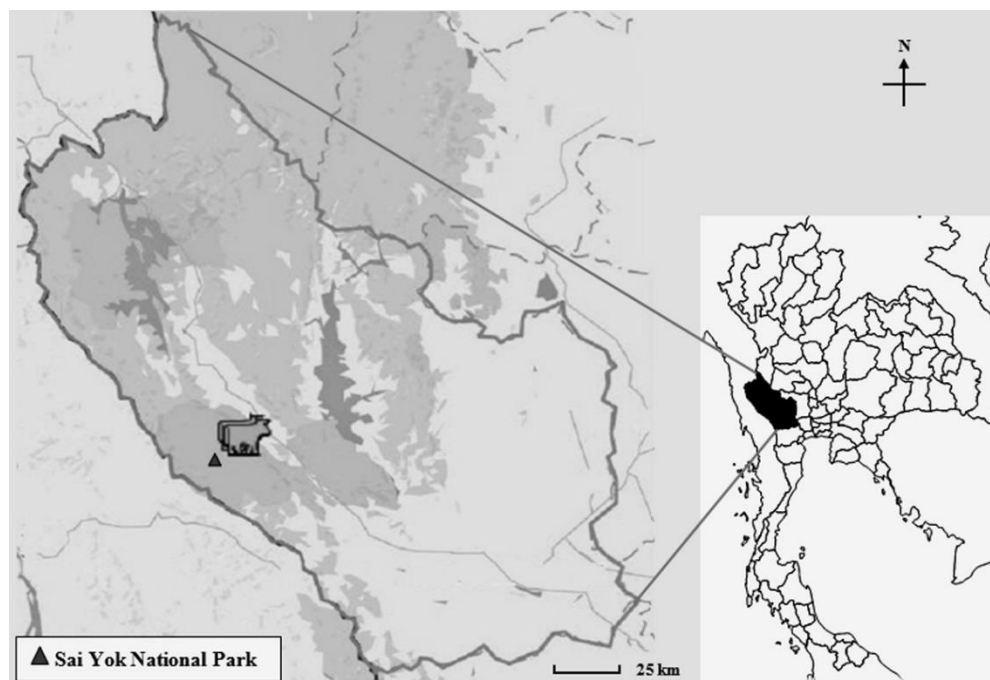
Hard ticks (Ixodidae) are obligate blood sucking arthropods of considerable medical and veterinary significance (Baneth, 2014). They transmit several protozoan, viral, bacterial, and fungal pathogens. Theileriosis and babesiosis are tick-borne protozoan diseases in livestock and wildlife (Michel et al., 2014; Zanet et al., 2014). These diseases have a major impact on livestock production, mainly on cattle and small ruminants in tropical and subtropical regions (Altay et al., 2008; Uilenberg, 1995). The protozoan parasites *Theileria parva* and *Theileria annulata* are highly pathogenic to cattle and cause lymphoproliferative disease, which has high mobility and mortality among susceptible animals. In contrast, other *Theileria* sp. (*Theileria orientalis*/*Theileria sergenti*/*Theileria buffeli*) are considered benign and cause mild or asymptomatic disease in cattle (Sarataphan et al., 1998). These parasites are transmitted by ticks of the genera *Rhipicephalus*, *Hyalomma*, *Amblyomma*, and *Haemaphysalis* (Bishop, 2004). Bovine babesiosis, which is mainly caused by *Babesia bovis* and *Babesia bigemina*, causes economic losses in the cattle industry. Both *Babesia* species are transmitted by cattle ticks *Rhipicephalus* (*Boophilus*) *microplus* and *Rhipicephalus annulatus* (Nicholson et al., 2009). It is known that *R. microplus* tick species infest cattle in Thailand (Changbunjong et al., 2009; Jittapalapong et al., 2004). A previous study confirmed infection by *B. bigemina* in *R. microplus* ticks collected from cattle in an enzootic area of Thailand (Jittapalapong et al., 2004). However, little is known about the circulation of tick-borne protozoan diseases in Thailand. The knowledge of pathogens transmitted by ticks in a given area is useful for assessing the risk of infection in animals.

The diagnosis of protozoan parasites has been based on traditional methods, which include Giemsa

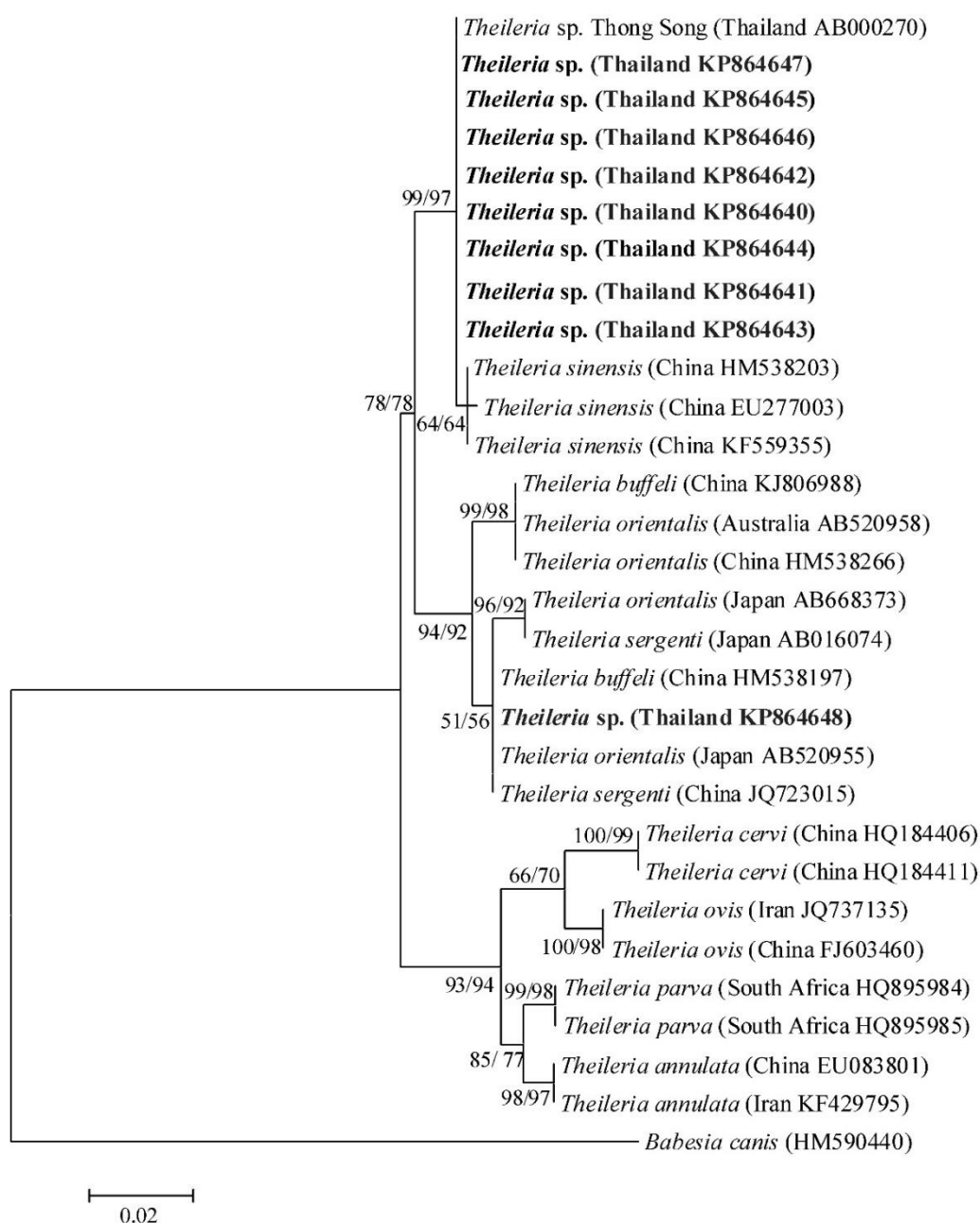
stained blood smears, serological assays, and observation of clinical symptoms. Currently, molecular diagnostic assays such as polymerase chain reaction (PCR) have been established as epidemiological and diagnostic tools for the detection and identification of the *Theileria* and *Babesia* species (Altay et al., 2008; Altangerel et al., 2011; Githaka et al., 2012; Zanet et al., 2014; El-Ashker et al., 2015). Many 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, is a suitable marker for detection and genetic characterization of blood parasites' DNA (Chansiri et al., 1999; Githaka et al., 2012). This study was carried out to detect tick-borne protozoa belonging to *Theileria* sp. and *Babesia* sp. in tick specimens collected from the buffer zone of Sai Yok National Park in Thailand and determine their phylogenetic relationship with other related species based on the 18S rRNA gene sequence.

## Materials and Methods

**Study sites:** From January to April 2014, an investigation was conducted in the buffer zone of Sai Yok National Park, Kanchanaburi Province, Thailand: N14°25'53.9'', E098°48'35.0'' and N14°25'08.02'', E098°48'36.76 (Fig 1). Buffer zones are defined as areas adjacent to protected areas that add to their protection and provide valuable benefits to neighboring rural communities (Sayer, 1991). Increasing numbers of people and their domestic livestock are living close to the national park boundary, and the exchange of disease between domestic animals and wildlife is becoming an increasingly serious problem. Therefore, the site may be a potentially important source of vector-borne diseases.



**Figure 1** Map of tick collection sites in the buffer zone of Sai Yok National Park.



**Figure 2** Phylogenetic analysis of the 18S rRNA gene of the *Theileria* species identified in this study and those present in the GenBank database. The numbers on branches indicate percent bootstrap support of neighbor-joining and maximum likelihood based on 1,000 replications.

**Tick collection:** Ticks were collected manually from 30 beef cattle (n = 25 cattle at the site 1 and 5 cattle at site 2) by their owners without identifying individual animals. They were preserved directly into 15 ml tubes containing 70% ethanol and sent to the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University, Thailand for further analysis. All specimens were identified based on morphological criteria under a stereomicroscope using taxonomic keys (Kohls, 1957). The ticks were pooled by sex and life stage ranged from 1 to 5.

**DNA extraction and 18S rRNA amplification:** Genomic DNA was extracted from individual engorged adult females, pools of adult females, and pools of adult males using a QIAamp DNA minikit

(QIAGEN, Germany) according to the manufacturer's protocol. The amplification of a 619 basepair (bp) fragment of the 18S rRNA gene was performed using PCR with the primer pair Ba/ThF 5' CCAATCCTGACACAGGGAGGTAGTGACA 3' was the forward primer and Ba/ThR 5'CCCCAGAACCCAAAGACTTTGATTCTCTCAA G 3' was the reverse primer (Kledmanee et al., 2009). The PCR was performed in a C1000 TM 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 s, annealing at 70°C for 45 s, extension at 72°C for 90 s, 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 GeneRuler™ 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). The sequencing reactions were analyzed using an ABI 3730 XL sequencer and fluorescent dye-terminator sequencing. DNA sequences were performed using Ba/ThF and Ba/ThR primers. All 18S rRNA sequence results were compared with the 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 neighbor-joining and maximum likelihood analysis with bootstrapping (1,000 replications) by MEGA 6 software (Tamura et al., 2013). The best nucleotide substitution model was a Tamura 3-parameter with a Gamma distribution parameter (T92+G model). All sequences were compared to published sequences from the GenBank database under the following accession numbers: KP864640-KP864648, AB000270, EU277003, KF559355, HM538203, KJ806988, HM538197, HM538266, AB520955, AB668373, AB520958, JQ723015, AB016074, HQ184406, HQ184411, FJ603460, JQ737135, HQ895984-HQ895985, EU083801 and KF429795. *Babesia canis* HM590440 was included as an outgroup.

## Results

In the present study, 10 (33.3%) out of 30 examined cattle were infested with ticks. A total of 85 ticks, including 51 adult females, 22 engorged adult females, and 12 adult males were collected from the infested cattle. Only one species of tick (*R. microplus*) was found in this study. The mean number of ticks per animals was 8.5. Nine (18%) out of 50 tick pools were positive for the 18S rRNA gene fragment of *Babesia/Theileria* species. PCR samples from the positive *R. microplus* ticks were sequenced; all samples were negative for *Babesia* sp., and all were identified as *Theileria* sp. From these nine *Theileria* sequences, eight shared 100% identity with the 18S rRNA gene of the unidentified *Theileria* species (*Theileria* type Thong Song AB000270) and 99% identity with *T. sinensis* (KF559355). The remaining sequence shared 99% identity with *T. orientalis/sergenti/buffeli* (AB520955, JQ723015 and HM538197). The phylogenetic relationships among the *Theileria* species infective to ticks can be divided into two groups (Fig 2). The *Theileria* sp. (KP864640-KP864647) identified in this study was included in the clade with the unidentified *Theileria* sp. (*Theileria* type Thong Song) and *T. sinensis*. The *Theileria* sp. (KP864648) was clustered with *T. orientalis/sergenti/buffeli*.

## Discussion

This study is the first to report on the cattle tick *R. microplus* infected with the bovine *Theileria* species in Thailand using molecular diagnostic assay. The results suggest ticks as a possible vector of bovine *Theileria* sp. Other genera of *Theileria* vectors such as *Amblyomma*, *Hyalomma*, and *Haemaphysalis* that have been identified in many countries (Bishop et al., 2004) were not found in this study. However, all of these except *Hyalomma* have been recorded in Thailand (Tanskul et al., 1983). In the present study, 33.3% of cattle were infested with ticks and only *R. microplus* was present on hosts, which is in accordance with previous research studies. Sarataphan et al. (1998) surveyed ticks in cattle in 25 provinces of Thailand and found the cattle tick *R. microplus* was dominant and had extensive distribution. Additionally, Changbunjong et al. (2009) found that more than 70% of cattle in Tak Province in western Thailand were infested with this type of tick. However, transmission of *Theileria* by *R. microplus* is unknown. Specific DNA of the *Theileria* species in our ticks may possibly be derived from host blood meal consumed from infected cattle. In our study, there was no vector potential of this tick was discovered. Hence, the detection of *Theileria* in unfed-ticks should be further investigated to determine their potential as a vector. In Thailand, the prevalence of the *Theileria* species has been detected in domestic animals. A study by Kaewthamasorn and Wongsamee (2006) found that 50% of beef cattle in Nan Province in northern Thailand were infected with the *Theileria* parasite. Additionally, Altangerel et al. (2011) demonstrated the prevalence of *T. orientalis* parasites circulating in the blood of cattle and water buffaloes in eight provinces of Thailand, with average percentages of 31.5% and 9.4%, respectively. In contrast, there are no reports of these parasites in the tick vectors in Thailand. In our study, the prevalence of bovine *Theileria* species in ticks was determined as 18%. The lack of detection of babesiosis in our study suggests that this infection may be not common in the surveyed area. However, further molecular studies targeting *Babesia* sp. are needed to determine whether ruminant babesiosis is present in this area. Diagnosis of blood protozoa can be made by microscopic examination of Giemsa stained blood smears and the presence of clinical symptoms in the acute phase of the disease, but after acute infection, recovered animals frequently sustain subclinical infections, which are microscopically undetectable (Esmaeilnejad et al., 2014). Although serological methods have been extensively used in the diagnosis of subclinical infections, false positive and false negative results are commonly observed (Altay et al., 2008). Therefore, molecular techniques have been developed and used as epidemiological and diagnostic tools for the detection and identification of parasites (Zanet et al., 2014; El-Ashker et al., 2015). In the present study, a molecular survey based on PCR amplification of the 18S rRNA gene was used for detection and genetic characterization of the *Theileria* and *Babesia* species. The results of PCR assays were confirmed by DNA sequencing. The sequences were further analyzed for genotypic identification. Our results identified two

different *Theileria* (*Theileria* sp. type Thong Song and the *T. orientalis/sergenti/buffeli* group). Both species of *Theileria* have been reported in Thailand (Chansiri et al., 1999; Altangerel et al., 2011). *Theileria* sp. type Thong Song was isolated from cattle from the south of Thailand and was classified as a benign *Theileria* species closely related to *T. sinensis*. According to the phylogenetic analysis, neighbor-joining and the maximum likelihood method provided highly similar results. Eight *Theileria* sp. (KP864640-KP864647) were included in the clade with unidentified *Theileria* species (*Theileria* type Thong song) and *T. sinensis*. *Theileria* sp. (KP864648) was clustered together with *T. orientalis/sergenti/buffeli*. All were different from known malignant *Theileria* species in cattle (*T. parva* and *T. annulata*) and benign *Theileria* species in cervids (*T. cervi*) and sheep (*T. ovis*). Our analyses confirmed and supported the results of previous studies showing the cluster groups of *Theileria* species based on phylogenetic studies of 18S rRNA, 28S rRNA, and the major piroplasm surface protein (MPSP) gene (Chansiri et al., 1999; Liu et al., 2010; Altangerel et al., 2011; Gou et al., 2013).

Several wild animals from many regions of the world have been reported as infested with *R. microplus*, including white-tailed deer (*Odocoileus virginianus*) (Busch et al., 2014), red deer (*Cervus elaphus*) (Rodríguez-Vivas et al., 2013), brown brocket deer (*Mazama gouazoubira*) (Silveira et al., 2011), pampas deer (*Ozotoceros bezoarticus*) (Cançado et al., 2009), banteng (*Bos javanicus*), and gaur (*Bos frontalis*) (Barré and Uilenberg, 2010). Recently, Sumrandee et al. (2015) revealed that *R. microplus* collected from samba deer in Khao Yai National Park in Thailand were infected with a *T. cervi*-like sp., which suggests that sambar deer are a reservoir host of the parasite. Furthermore, many blood protozoan species of domestic animals such as *Theileria* sp., *Babesia bovis*, and *Babesia bigemina* have been reported in wild cervids (Silveira et al., 2011). Therefore, when domestic and wild animals are in close contact and vectors are present in the area, diseases could possibly be exchanged among them.

In conclusion, this study revealed two *Theileria* (*Theileria* sp. type Thong Song and *T. orientalis/sergenti/buffeli*) in ticks. Our results provide important information about the distribution and genetics of blood parasites in ticks collected from cattle at the buffer zone of Sai Yok National Park that can be used for prevention and control measures to reduce economic losses.

### Acknowledgements

This work was supported by grant from Faculty of Veterinary Science, Mahidol University. We would like to thank the staffs of The Monitoring and Surveillance Center for Zoonotic Disease in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University, for supplying specimens.

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## บทคัดย่อ

### การใช้วิธีทางอนุชีวโมเลกุลตรวจหาเชื้อโปรโตซัวในเลือดในเห็บที่เก็บจากโคในพื้นที่แนวกันชน ของอุทยานแห่งชาติไทรโยค ประเทศไทย

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เห็บแคงเป็นพาหะถ่ายทอดเชื้อโรคหลายชนิดในสัตว์และมนุษย์ การศึกษานี้ได้ดำเนินการตรวจหาเชื้อโปรโตซัว (*Theileria* และ *Babesia*) ในเห็บ ที่เก็บจากโคเนื้อในพื้นที่แนวกันชนของอุทยานแห่งชาติไทรโยค ประเทศไทย โดยได้ทำการตรวจหาเห็บจากโคจำนวน 30 ตัว พบว่ามีโคจำนวน 10 ตัว (ร้อยละ 33.3) ที่พบเห็บ เห็บที่เก็บได้ทั้งสิ้นมีจำนวน 85 ตัว จำแนกชนิดเป็น *Rhipicephalus* (*Boophilus*) *microplus* เห็บทั้งหมดถูกแบ่งออกเป็น 50 ตัวอย่างจำแนกตามเพศและระยะของเห็บ จากนั้นตัวอย่างถูกนำไปวิเคราะห์ด้วยกระบวนการปฏิกิริยาลูกโซ่โพลีเมอเรส (PCR) ซึ่งเป็นการเพิ่มจำนวนยีนของ 18S ไรโบโซมอล อาร์เอ็นเอ (rRNA) เพื่อตรวจหาเชื้อ *Theileria* และ *Babesia* ผลการศึกษาพบว่า 9 ตัวอย่าง (ร้อยละ 18) ตรวจพบเชื้อ *Theileria* โดยกลุ่มนี้ 8 ตัวอย่าง (ร้อยละ 88.9) มีความเหมือนกับ *Theileria* ที่ยังจำแนกชนิดไม่ได้ร้อยละ 100 ขณะที่อีก 1 ตัวอย่าง (ร้อยละ 11.1) มีความเหมือนกับ *T. orientalis/sergenti/buffeli* ร้อยละ 99 จากการวิเคราะห์ความสัมพันธ์เชิงวิวัฒนาการพบว่า *Theileria* ที่ยังจำแนกชนิดไม่ได้มีความใกล้ชิดกับ *T. sinensis* และแยกออกไปจากเชื้อ *Theileria* ชนิดอื่นๆ อย่างชัดเจน การศึกษานี้เป็นการรายงานครั้งแรกของการตรวจพบเชื้อ *Theileria* ของโค ในเห็บชนิด *R. microplus* โดยใช้เทคนิคทางอนุชีวโมเลกุลในประเทศไทย

**คำสำคัญ:** *Rhipicephalus microplus*, ไรโบโซมอล อาร์เอ็นเอ, *Theileria*, เห็บ

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