Incidences of hemoparasitic infections in cattle from central and northern Thailand

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Background: Hemoparasites, such as *Babesia* spp., *Theileria* spp. and *Anaplasma* spp., can negatively affect the health of farm animals resulting in significant losses in production. These losses inherently affect the economics of the livestock industry. Many blood parasitic diseases are recognized as being zoonosis. Since increases in the severity of vector-borne diseases in the southeast Asian region have been reported, investigations of parasitic epidemiology in Thailand will be necessary to improve the existing parasite-control strategies for blood parasitic infections. This study aims to investigate incidences of bovine hemoparasites throughout central and northern Thailand by focusing on areas of high density cattle populations.

Methods: Blood parasitic infections among cattle were screened and identified by microscopic examination. Anemia status was then determined by evaluation of the packed cell volume (PCV) of each animal. Furthermore, blood parasites were detected and identified by genus and species-specific primers through the polymerase chain reaction method. Amplicons were subjected to DNA sequencing; thereafter, phylogenetic trees were constructed to determine the genetic diversity and relationships of the parasite in each area.

Results: A total of 1,066 blood samples were found to be positive for blood parasitic infections as follows: 13 (1.22%), 389 (36.50%), and 364 (34.15%) for *Babesia bovis*, *Theileria orientalis*, and *Anaplasma marginale*, respectively. Furthermore, multiple hemoparasitic infections in the cattle were detected. The hematocrit results revealed 161 hemoparasitic infected samples from 965 blood samples, all of which exhibiting indications of anemia with no significant differences. Sequence analysis of the identified isolates in this study revealed that *B. bovis rap-1*, four separate clades of *T. orientalis msps*, and *A. marginale msp4* exhibited homology with other isolates obtained from different countries in ranges between 98.57–100%, 83.96-100%, and 97.60–100%, respectively. Conclusion: In this study, the analyzed incidence data of cattle hemoparasitic infection in Thailand has provided valuable and basic information for the adaptation of blood-borne parasitic infections control strategies. Moreover, the data obtained from this study would be useful for future effective parasitic disease prevention and surveillance among cattle.

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Abstract

Background: Hemoparasites, such as *Babesia spp.*, *Theileria spp.,* and *Anaplasma spp.*, can negatively affect the health of farm animals resulting in significant losses in production. These losses can inherently affect the economics of the livestock industry. Many blood parasitic diseases are recognized as being zoonosis. Since increases in the severity of vector-borne diseases in the southeast Asian region have been reported, investigations of parasitic epidemiology in Thailand will be necessary to improve existing parasite-control strategies for blood parasitic infections. This study aims to investigate incidences of bovine hemoparasites throughout central and northern Thailand by focusing on areas of high density cattle populations.

Methods: Blood parasitic infections among cattle were screened and identified by microscopic examination. Anemia status was then determined by evaluation of the packed cell volume (PCV) of each animal. Furthermore, blood parasites were detected and identified by genus and species-specific primers through the polymerase chain reaction method. Amplicons were subjected to DNA sequencing; thereafter, phylogenetic trees were constructed to determine the genetic diversity and relationships of the parasites in each area.

Results: A total of 1,066 blood samples were found to be positive for blood parasitic infections as follows: 13 (1.22%), 389 (36.50%), and 364 (34.15%) for *Babesia bovis, Theileria orientalis,* and *Anaplasma marginale*, respectively. Furthermore, multiple hemoparasitic infections in the cattle were detected. The hematocrit results revealed 161 hemoparasitic infected samples from 965 blood samples, all of which exhibited indications of anemia with no significant differences. Sequence analysis of the identified isolates in this study revealed that *B. bovis rap-1*, four separate clades of *T. orientalis msps,* and *A. marginale msp4* exhibited homology with other

isolates obtained from different countries in ranges between 98.57–100%, 83.96-100%, and 97.60–100%, respectively.

Conclusion: In this study, the analyzed incidence data of cattle hemoparasitic infection in Thailand has provided fundamental but valuable information for the adaptation of blood-borne parasitic infection control strategies. Moreover, the data obtained from this study will be useful in developing future effective parasitic disease prevention and surveillance protocols for the cattle industry.

Keywords: Hemoparasites, *Babesia spp.*, *Anaplasma spp.*, *Theileria spp.,* Cattle, Thailand

Introduction

Bovine hemoparasitic diseases, such as babesiosis, theileriosis, and anaplasmosis, are widely distributed throughout tropical and sub-tropical regions including Thailand. Most of these hemoparasitic diseases are actually tick-borne diseases that can adversely impact animal health, the livestock industry, and on occasion, human beings. Infections can be deadly to farm animals but are also known to be the cause of fever, anorexia, jaundice, increased abortion rates, and sterility, all of which can lead to reduced levels of milk and meat production (*Abdullah et al. 2019*). Bovine babesiosis is a serious challenge to the health of farm animals and is caused by a protozoan parasite of the genus *Babesia* that is found in the erythrocyte. Two species, *Babesia bovis* and *Babesia bigemina*, are known to be extremely prevalent worldwide via their geographical distribution (*Bock et al. 2004; Sawczuk 2007*), while other species, such as *Babesia divergens*, *Babesia major*, *Babesia jakimovi*, *Babesia ovata*, *Babesia occultans*, and *Babesia mymensingh*, have also been implicated in cattle infections (*Sivakumar et al. 2018; Chauvin et al. 2009*).

Bovine theileriosis is a hemoparasitic disease caused by protozoans of the genus *Theileria*. This protozoan is found in the blood and lymphatic systems of infected animals. *Theileria orientalis, Theileria annulata*, *Theileria parva*, *Theileria taurotragi*, and *Theileria velifera* are known to be the cause of bovine theileriosis (*Abdullah et al. 2019; Olds et al. 2018*). *Theileria annulata* and *Theileria parva* are highly virulent lympho-proliferative parasites that cause tropical theileriosis and East Coast fever, respectively (*Mukhebi et al. 1992*). *Theileria orientalis* is a non-lymphoproliferative *Theileria* parasite that is widely distributed throughout Southeast Asia (*Kamau et al. 2011; Mcfadden et al. 2011*).

Bovine anaplasmosis is another tick-borne disease caused by a rickettsia of the *Anaplasmataceae* family. *Anaplasma marginale*, *Anaplasma phagocytophilum*, and *Anaplasma centrale* are important species that are known to infect cattle (*Kocan et al. 2000; Hornok et al. 2007*). *Anaplasma marginale* is known to be the most prevalent tick-borne parasite of cattle worldwide (*Kocan et al. 2010*). Accordingly, there have been many reports of *Babesia spp., Theileria spp.,* and *Anaplasma spp.* co-infections in cattle (*Altay et al. 2008; Suarez et al. 2011; Bursakov et al. 2019; Nyabongo et al. 2021; Zhou et al. 2019*).

The occurrence of bovine hemoparasitic infection has been reported in different parts of Thailand (*Sukhumsirichart et al. 1999; Altangerel et al. 2011; Simking et al. 2013; Jirapattharasate et al. 2016; Jirapattharasate et al. 2017*). Hence, this study investigated the parasitic epidemiology of north and central Thailand to improve the general understanding of these infections and to contribute towards effective efforts of strategic control.

Materials & Methods

Sample and data collection

This study was conducted between June, 2020 and April, 2021. Dairy cattle farms and beef cattle farms with high population densities that were located in six provinces in northern and central Thailand were selected for this study. The sample-collection protocols were reviewed and approved of by the Animal Care and Use Committee at Faculty of Veterinary Medicine, Chiang Mai University (Project no.R000028479, Approval No. S26/2563). Farm owner permission letters were distributed and agreed to before samples were collected. The provinces included in this study were Chiang Mai (n=143), Chiang Rai (n=87), Lamphun (n=557), Lampang (n=76), Phayao (n=122), and Nakhon Pathom (n=81) (Figure1). A total of 1,066 blood samples were collected from randomly selected farms located in five provinces in northern Thailand and another province in central Thailand. The animals were restrained and blood was collected from their coccygeal or jugular veins and immediately transferred into EDTA-K2 lyophilized vacuum blood collection tubes (BD Vacutainer®, Franklin Lakes, NJ, USA). The blood sample tubes were kept in a cooled box equipped with ice packs during transport to the Faculty of Veterinary Medicine, Chiang Mai University and were then processed immediately. Data related to the characteristics of the animals according to farm management were obtained and recorded by the investigators. At each farm, farm owners or farm staff were interviewed with regard to specific individual animal characteristics, namely age, breed, and gender. Farm-based characteristics included location, history of hemoparasitic infections, treatment details, tick control programs, and farm management practices.

Microscopic analysis

A thin smear of blood was collected from each blood sample. The blood smears were air-dried, fixed in methanol for two minutes, and stained by 10% Giemsa solution (Merck,

Kenilworth, NJ, USA) in phosphate-buffered saline. The smears were examined at 1000X magnification using an oil-immersion lens (Olympus CX31, Shinjuku City, Tokyo, Japan). The identification process was carried out to decipher genus and species profiling to the greatest degree possible. A minimum of 1,000 red blood cells were counted and recorded. The percent of parasitemia was determined by counting the number of infected red blood cells (iRBCs) and by then dividing that number by the total number of red blood cells (RBCs): % parasitemia = (iRBCs/RBCs) × 100.

DNA extraction from blood

Genomic DNA was extracted from all blood samples using a PureLink™ Geomic DNA mini kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. The extracted DNA was eluted in 70 µL of the elution buffer. The quantity and quality of the DNA were determined with the use of an UV/Vis spectrophotometer DU 730 (Beckman Coulter, Brea, CA, USA). The DNA was stored at -20°C until it was used.

Packed Cell Volume determination using microhematocrit method

Blood samples were pipetted into capillary tubes and spun in a high-speed centrifuge. After five minutes of centrifugation, hematocrit results were estimated by calculating the ratio of the column of packed erythrocytes to the total length of the sample in the capillary tube and then measured with a graphic reading pad. The reference value was determined to be between 24 and 46 percent (*Miller et al. 1989*).

PCR detection of cattle hemoparasites

For the purposes of PCR analysis, *Babesia spp.* and *Theileria spp.* parasites were screened for the presence of genetic differences among *Babesia* 18S rRNA (*Hilpertshauser et al. 2006*) and *Theileria spp*. 18S rRNA (*Cao et al. 2013*). Positive control samples were included for each specific screen. *Babesia bovis* and *Babesia bigemina* were detected among *B. bovis* rhoptry-associated protein (*rap-1*) (*Figueroa et al. 1993*), *B. bigemina* apical membrane antigen 1 (*ama-1*) (*Sivakumar et al. 2012*), *T. orientalis* major piroplasm surface protein (*mpsp*) (*Ota et al. 2009*), and *T. annulata* 30 kDa major merozoite surface antigen gene (*tams-1*) (*Kirvar et al. 2000*). Furthemore, the prevalence of *Anaplasma marginale* and *Anaplasma phagocytophilum* was determined by genetic variations of *A. marginale* major surface protein 4 (*msp*4) (*M'ghirbi et al. 2016*) and *A. phagocytophilum* major surface protein 2 (*msp*2) (*M'ghirbi et al. 2016*), respectively.

DNA from each sample was PCR amplified using the primer specific gene sequences listed in Table 1. *Babesia spp.* and *Theileria spp.* parasites were further screened using nested PCR (nPCR). Positive samples were specifically screened, while *Babesia bovis* and *Babesia bigemina* were screened by nPCR. Meanwhile, *Theileria orientalis, Theileria annulata, Anaplasma marginale,* and *Anaplasma phagocytophilum* parasites were screened by primary PCR. The final volume of 30 µl was comprised of 5 µl of template DNA, 25 µl of the reaction mixture with 2X MyTaq HS Red Mix (Meridian Bioscience, Bioline, Memphis, TN, USA), and 10 µM of each sample. Deionized water was then added as needed to reach the final desired volume.

The conditions used for *Babesia spp.* and *Theileria spp.* amplification consisted of an initial denaturing step at 95 °C for 1 min, 35 cycles of a denaturing step at 95 °C for 15 sec, an annealing step for *Babesia spp.* at 63 °C for 15 sec and *Theileria spp.* at 55 °C for 30 sec, an

extension step at 72 °C for 15 sec, and a final extension step at 72 °C for 30 sec. The same concentration of MyTaq HS Red Mix was used for *Theileria spp.* amplification at 5 µL of the PCR product in the nPCR, as has been described above. The nPCR condition included an initial denaturation step at 95 °C for 1 min and 35 cycles of a denaturing step at 95 °C for 15 sec, annealing temperatures of 60 °C for 10 sec for *Babesia spp* with an extension step at 72 °C for 10 sec, and a final extension step at 72 °C for 30 sec. Then, *Babesia spp.* positive samples were identified for *B. bovis* and *B. bigemina.* The same concentration of MyTaq HS Red Mix that was used for amplification consisted of an initial denaturing step at 95 °C for 1 min, 35 cycles of a denaturing step at 95 °C for 15 sec, an annealing step for *B. bovis* at 55 °C for 15 sec and *B. bigemina* at 61 °C for 15 sec, an extension step at 72 °C for 15 sec, and a final extension step at 72 °C for 30 sec. The same conditions used for *B. bovis* and *B. bigemina* amplification at 5 µl of the PCR product in the nPCR were used for the first PCR of each strain. Accordingly, *Theileria spp.* positive samples were identified for *T. orientalis* and *T. annulata.* Identification of *T. orientalis* and *A. phagocytophilum* was performed in a PCR thermal cycler consisting of an initial denaturing step at 94 °C for 3 min, 40 cycles of a second denaturing step at 94 °C for 1 min, an annealing step at 58 °C for 30 sec, an extension step at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. Similarly, in terms of the PCR specification of *A. marginale* and *T. annulata.*, the initial denaturing step was set at 94 °C for 3 min, 40 cycles of a second denaturing step at 94 °C for 1 min, an annealing step at 60 °C for 30 sec, an extension step at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. All PCR products were separated by gel electrophoresis on 1% agarose in 1X TAE buffer and visualized using ethidium bromide under UV transilluminator.

185 **DNA sequencing and phylogenetic tree analysis**

186 *B. bovis* (n = 4), *T. orientalis* (n = 12), and *A. marginale* (n =5) positive samples collected
187 from each province were randomly selected for DNA sequencing. PCR products were purified
188 using a PureLink^{TM} quick PCR purification kit (Invitrogen, Thermo Fisher Scientific, Waltham,
189 MA, USA). The purified PCR samples were sent to ATCC Co.Ltd. (Thailand Science Park,
190 Thailand) for identification of species by DNA sequencing. Nucleotide sequences were analyzed
191 using the BLAST tool on Clustal 2.1 software. The completed sequences were subjected to
192 multiple sequence alignment with sequences previously available in the GenBank database.
193 Phylogenetic trees in this study were analyzed using MEGA X program. Accordingly, *Rap-1*
194 gene sequences of *B. bovis* (n = 4), *mpsp* gene sequences of *T. orientalis* (n = 12), *msp4* gene
195 sequences of *A. marginale* (n =5), and those reported from other regions were used to construct a
196 subsequent phylogenetic tree. A bootstrap test with 2,000 replications was used to establish the
197 confidence of the branching pattern of the trees. Finally, the phylogenetic relationship among the
198 isolates identified in this study and those isolated from different countries were illustrated.
199

200 **Statistical analysis**

201 Statistical analysis of data categorized as positive or negative for *B. bovis, T. orientalis,*
202 and *A. marginale* was accomplished based on PCR results and performed using the chi-square
203 test. A *P*-value of < 0.05 was considered to be statistically significant using GraphPad Prism
204 version 8.4.
205

206 **Results**

207 **Microscopic examination of cattle hemoparasitic infections**

208 According to light microscopic examinations, variable cattle hemoparasites, such as
209 *Babesia Spp.*, *Theileria spp.,* and *Anaplasma spp.*, were detected in Giemsa-stained blood smears
210 (Figure 2). Paired-pyriform parasites within the erythrocyte were observed explicitly as a
211 characteristic of *Babesia Spp.* The pyriform shape of the *Theileria* parasites was clearly detected.
212 A small spot located on the edge or center of the red blood cell was confirmed as a characteristic
213 indicator of *Anaplasma spp.*
214
215 **Hematological examination**
216 Accordingly, 965 blood samples out of a total of 1,066 samples were examined. The
217 results indicated that 161 hemoparasite infected samples exhibited positive indications of
218 anemia, as is shown in Table 2.
219
220 **Molecular detection and identification of cattle hemoparasites**
221 Using the specific primers, PCR products at 298 bp, 776 bp, and 420 bp were determined
222 to represent *B. bovis, T. orientalis*, and *A. marginale*, respectively (Figure 3). Meanwhile, *B.*
223 *bigemina, T. annulata,* and *A. phagocytophilum* were not detected. The PCR results indicated
224 that 1.22% (13/1,066) of the blood samples were positive for *B. bovis, T. orientalis,* and *A.*
225 *marginale* at 36.50% (389/1,066) and 34.15% (364/1,066), respectively as is shown in Table 3.
226 Furthermore, multiple infections of two or more cattle hemoparasites appeared in 27.30 %
227 (291/1,066) of the total blood samples (Figure 4). By the presence of multiple infections, *T.*
228 *orientalis* (99.66 %, 290/291) was found to be the most frequent hemoparasite.
229
230 **DNA sequencing and phylogenetic analysis of cattle hemoparasites**

The molecular characterizations of cattle hemoparasites were analyzed with respective gene targets of *B. bovis rap -1, T. orientalis mpsp*, and *A. marginale msp4*. The identity of the *B. bovis* specificity among isolates in this study ranged between 98.57–100%. Four isolates obtained from Lamphun (OK490920), Lampang (OK490921 and KO490922), and Nakhon Pathom (OK490919) Provinces with a product size of 298 bp that shared a degree of similarity with the isolates obtained from China (KT312809.1) and the Philippines (JX860283.1) (Figure 5).

Further characterizations of *T. orientalis* and *A. marginale* were identified using phylogenetic analysis with PCR assay. A product size of 776 bp in this study was placed in four isolated genotypes using percent identities ranging between 83.96–100% (Figure 6). The phylogenetics of six isolates obtained from Lamphun (OK490929), Lampang (OK490926 and OK490928), Phayao (OK490927 and OK490930), and Nakhon Pathom (OK490931) Provinces were related to *T. orientalis* type 5 and shared a degree of similarity with isolates obtained from several other areas on the Asian continent. There were two isolates obtained from Phayao (OK490923) and Nakhon Pathom (OK490924) Provinces that were related to the *T. orientalis* type 3 and shared similarity with isolates obtained from Sri-Lanka (AB701465) and Vietnam (AB560821). Moreover, another sequence result obtained from Lamphun (OK490925) Province revealed the presence of *T. orientalis* type 4, which has been reported to be present in China (MH539832), Myanmar (AB871316), and Thailand (AB562561). *T. orientalis* type 7 was found lastly in this study, while three isolates from Chiang Mai (OK490933), Chiang Rai (OK490934), and Nakhon Pathom (OK490932) Provinces exhibited similarity with the databases established from Japan (AB218430), China (MH539826), Indonesia (AF102500), and Vietnam (AB560823).

253 Finally, a PCR product size of 420 bp confirmed the presence of *A. marginale*. The
254 phylogenetic findings of five isolates obtained from Lamphun (OK506074, OK506075, and
255 OK506077), Chiang Rai (OK506076), and Nakhon Pathom (OK506073) Provinces revealed
256 97.60–100% of genetic homology when compared to isolates obtained from Brazil (JN022561),
257 Columbia (MF771065), and South Africa (KF758944 and MT173811) (Figure 7).
258

259 ## Discussion

260 Thailand is known to be an endemic area for various bovine tick-borne pathogens, which
261 can affect the health of farm animals and result in significant economic losses to the livestock
262 industry. Incidence studies involving cattle hemoparasitic infections could provide researchers
263 with valuable information and contribute towards the development of effective efforts for
264 strategic control. In this study, microscopic analysis, which is the worldwide standard protocol,
265 was performed for primary detection of these parasites. However, parasitemia has been found to
266 be very low, while morphological differentiations of various *Theileria spp.* and *Babesia spp.* ring
267 forms were inconclusive. Consequently, molecular tools are needed to verify complementary
268 diagnostic information with a high degree of specificity and sensitivity.
269 *Babesia spp*. and *Theileria spp.* were screened. Afterward, the specific genus of each
270 parasite, *Babesia bovis, Theileria orientalis*, and *Anaplasma marginale*, was determined by
271 specific screening. In the present study, the positive rates of those hemoparasites were variable
272 for the different sampling sites in Thailand. Importantly, the sampling period, tick control
273 program, and farming management practices were also relevant to the positive results. Although
274 the hematocrit results detected anemia in the hemoparasitic infected samples, no significant
275 differences were observed (Table 2). It is important to recognize that hemoparasitic infected

276 animals can go undetected as they may be tested during the parasitic incubation period, which
277 could then result in low parasitemia, an absence of clinical signs, and the interpretation of normal
278 hematocrit results. Consequently, the reported percent of hematocrit may possibly be related to
279 the dehydration of the animal.

280 Previous epidemiological studies conducted in Thailand have helped to identify and
281 manage the relevant burden and risk factors associated with incidences of tick-borne diseases
282 (*Jirapattharasate et al. 2016; Jirapattharasate et al. 2017*). In this study, PCR was used as a
283 specific tool for *B. bovis*, *T. orientalis,* and *A. marginale* detection because it has been reported
284 to be a highly specific and sensitive method (*Altay et al. 2008*). Overall, the sampled cattle had at
285 least one incidence of hemoparasite (*B. bovis* (1.22%), *T. orientalis* (36.50%), and *A. marginale*
286 (34.15%)) infections. These detections were not significantly different from those of previous
287 studies conducted in Thailand, wherein the prevalence of the above parasites ranged from 0.8 to
288 31.0% (*Altangerel et al. 2011; Jirapattharasate et al. 2017; Sarataphan et al. 2003*).
289 Furthermore, there have been some reports of a female tick vector, *Rhipicephalus (Boophilus)*
290 *microplus*, which has exhibited a higher frequency of infection with *B. bigemina* than *B. bovis.*
291 Hence, the chance of *B. bigemina* transmission by the tick vector is higher than *B. bovis*
292 (*Oliveira-Sequeira et al. 2005; Oliveira et al. 2008*). Although, previous studies have reported a
293 higher occurrence of *B. bigemina* than *B. bovis* (*Jirapattharasate et al. 2016; Jirapattharasate et*
294 *al. 2017*), *B. bigemina* went undetected in this study. This might be due to the increased drug
295 resistant prowess of *B. bovis* when compared to *B. bigemina*. The sequence analysis of the *B.*
296 *bovis* apical membrane antigen 1 (BbAMA-1) obtained from Thai cattle has exhibited a low level
297 of polymorphism among global isolates, while some epitopes were found to infrequently be
298 polymorphic due to amino acid mutations (*Rittipornlertrak et al. 2017*). This problem is

299 indicative of the challenges associated with this vaccine candidate and the process of novel
300 antibabesial drug development. According to interviews conducted with farmers in this study, the
301 tick control program was especially noteworthy. The cattle at most sampling farms were treated
302 with diminazene aceturate and ivermectin in order to prevent hemoparasitic infection and to
303 avoid establishing a parasite vector. Even though this practice might increase the drug resistance
304 index (*Chitanga et al. 2011; Tuvshintulga et al. 2019; Chaparro-Gutiérrez et al. 2020*), it is
305 currently a widely-used as an element of a pervasive tick control program and farming
306 management practice in Thailand. From the hemoparasite detection results, diminazene aceturate
307 and ivermectin seemed capable of preventing some incidences of cattle hemoparasites. We found
308 minimal *Babesia spp.* infections at all sampling farms, whereas *Theileria spp.* and *Anaplasma*
309 *spp.* infections remained high. Notably, *T. orientalis* was recognized as the most frequently
310 identified hemoparasite with multiple infections (99.66 %). Detection of this hemoparasite is
311 evidence of the need to develop a combined vaccine or drug for the treatment of multi-
312 hemoparasitic infection.

313 Based on DNA sequencing and the phylogenetic tree findings, *B. bovis rap-1* was highly
314 conserved amongst the cattle samples in the current study and exhibited a high correlation with
315 other previously reported geographic isolates. These results confirm that the *rap-1* gene is a
316 useable target for the detection of hemoparasites from different geographic areas (*Figueroa et al.*
317 *1993*). The phylogenetic tree of *B. bovis* isolates in these three provinces indicated that the *rap-1*
318 gene is relatively conserved. It appears that *B. bovis* isolates obtained from northern and central
319 Thailand were of the same strain as other geographic areas. Although, Nakhon Pathom is located
320 in central Thailand, the *rap-1* gene isolate obtained from this location was identified with isolates
321 collected from northern provinces, namely Lamphun and Lampang, as these areas are located on

the same continent as China and the Philippines. Hence, an effective approach for disease tracking will be beneficial as a control strategy for bovine babesiosis in these locations.

Moreover, phylogenetic analysis in this study also revealed that *T. orientalis mpsp* gene sequences were classified into 4 clades (type 3, type 4, type 5, and type 7), which was similar to the findings of a previous report (*Altangerel et al. 2011; Jirapattharasate et al. 2017*). This result confirmed that the *mpsp* gene is a highly polymorphic gene that exhibited a wide range of diversity among the different filed isolates (*Sivakumar et al. 2014*). In this study, we also found that cattle from every sampling farm were positive for *Theileria spp.* infection. It could then be inferred that *Theileria spp.* infection is commonly found in these areas. Therefore, effective farm management practices and routine tick control campaigns (*L'hostis et al. 2002*) would help to reduce the prevalence of bovine Theileriosis and other tick-borne parasitic diseases in northern and central Thailand.

According to existing genetic diversity, the nucleotide sequence levels of *A. marginale* were based on the *msp4* gene. Sequences of the *msp4* gene obtained in this study were conserved and aligned with those of previous reports (*Junsiri et al. 2020*). Phylogenetic analysis revealed that all the *msp4* sequences were clustered with sequences obtained from Brazil, Columbia, Portugal, and South Africa. Previous reports on animal movement also suggest that the genetic diversity of *A. marginale* in this study correlated to incidences of *A. marginale* infection in various other countries (*Jirapattharasate et al. 2017*). Therefore, restricting animal transportation may help to effectively control the genetic diversity of *A. marginale* and other hemoparasites.

Conclusions

345 The distribution of bovine hemoparasites across a wide geographical area of northern and
346 central Thailand has revealed that *T. orientalis* is an endemic hemoparasite among Thai cattle.
347 However, *B. bovis* detection rates appeared to decrease when compared with those of previous
348 reports. While *A. marginale* is a highly prevalent pathogen in cattle from the north and central
349 regions of Thailand, these findings can improve the general understanding of the epidemiology
350 of hemoparasites in Thailand and can contribute to the design of effective parasite control
351 strategies in the future.
352

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355 providing the necessary laboratory facilities. In addition, we thank all owners and members of
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357

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359

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474

Table 1(on next page)

Forward and reverse primers used for the detection of cattle hemoparasitic infection.

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1 **Table 1.** Forward and reverse primers used for the detection of cattle hemoparasitic infection.

Species	Target gene	Oligonucleotide sequence (5́→ 3́)	Size (bp)	Ref.
Babesia spp.	18S rRNA	Outer forward: GTTTCTGMCCCATCAGCTTGAC	1201-1248	(*Hilpertshauser et al. 2006*)
		Outer reverse: GCATACTAGGCATTCCTCGTTCAT		
		Inner forward: GTTTCTGMCCCATCAGCTTGAC	494-528	
		Inner reverse: CAACCGTTCCTATTAACCATTAC		
B. bovis	*rap -1*	Outer forward: CACGAGGAAGGAACTACCGATGTTGA	365	(*Figueroa et al. 1993*)
		Outer reverse: CCAAGGAGCTTCAACGTACGAGGTCA		
		Inner forward: TCAACAAGGTACTCTATATGGCTACC	298	
		Inner reverse: CTACCGAGCAGAACCTTCTTCACCAT		
B. bigemina	*ama-1*	Outer forward: TCGGCAGGTGCTCTTACAAAC	711	(*Sivakumar et al. 2012*)
		Outer reverse: GTTCAGGATACGGCAAACACC		
		Inner forward: ATTTGTCGCCAGTATCAGCCG	480	
		Inner reverse: CAATGTCAACATCCGCAGCTG		
Theileria spp.	18S rRNA	Outer forward: GAAACGGCTACCACATCT	778	(*Cao et al. 2013*)
		Outer reverse: AGTTTCCCCGTGTTGAGT		

		Inner forward: TTAAACCTCTTCCAGAGT	581	
		Inner reverse: TCAGCCTTGCGACCATAC		
T. orientalis	*mpsp*	CTTTGCCTAGGATACTTCCT	776	(*Ota et al. 2009*)
T. annulata	*tams-1*	ATGCTGCAAATGAGGAT	785	(*Kirvar et al. 2000*)
A. marginale	*msp4*	ATCTTTCGACGGCGCTGTG	420	(*M'ghirbi et al. 2016*)
A. phagocytophilum	*msp2*	CCAGCGTTTAGCAAGATAAGAG	334	(*M'ghirbi et al. 2016*)

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Table 2(on next page)

Packed Cell Volume determination by the microhematocrit method.

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Table 2. Packed Cell Volume determination by the microhematocrit method.

Species	Packed Cell Volume	Hemoparasitic infection		Chi square	P-value
		positive	negative		
Babesia spp.	< 24 %	3	127	0.01221	0.9120
	24 – 46 %	18	817		
Theileria spp.	< 24 %	103	27	3.187	0.0742
	24 – 46 %	599	236		
Anaplasma spp.	< 24 %	55	75	1.409	0.2352
	24 – 46 %	308	527		

Table 3(on next page)

Summary of PCR screening results for *B. bovis, T. orientalis*, and *A. marginale* single infections in cattle from the northern and central Thailand.

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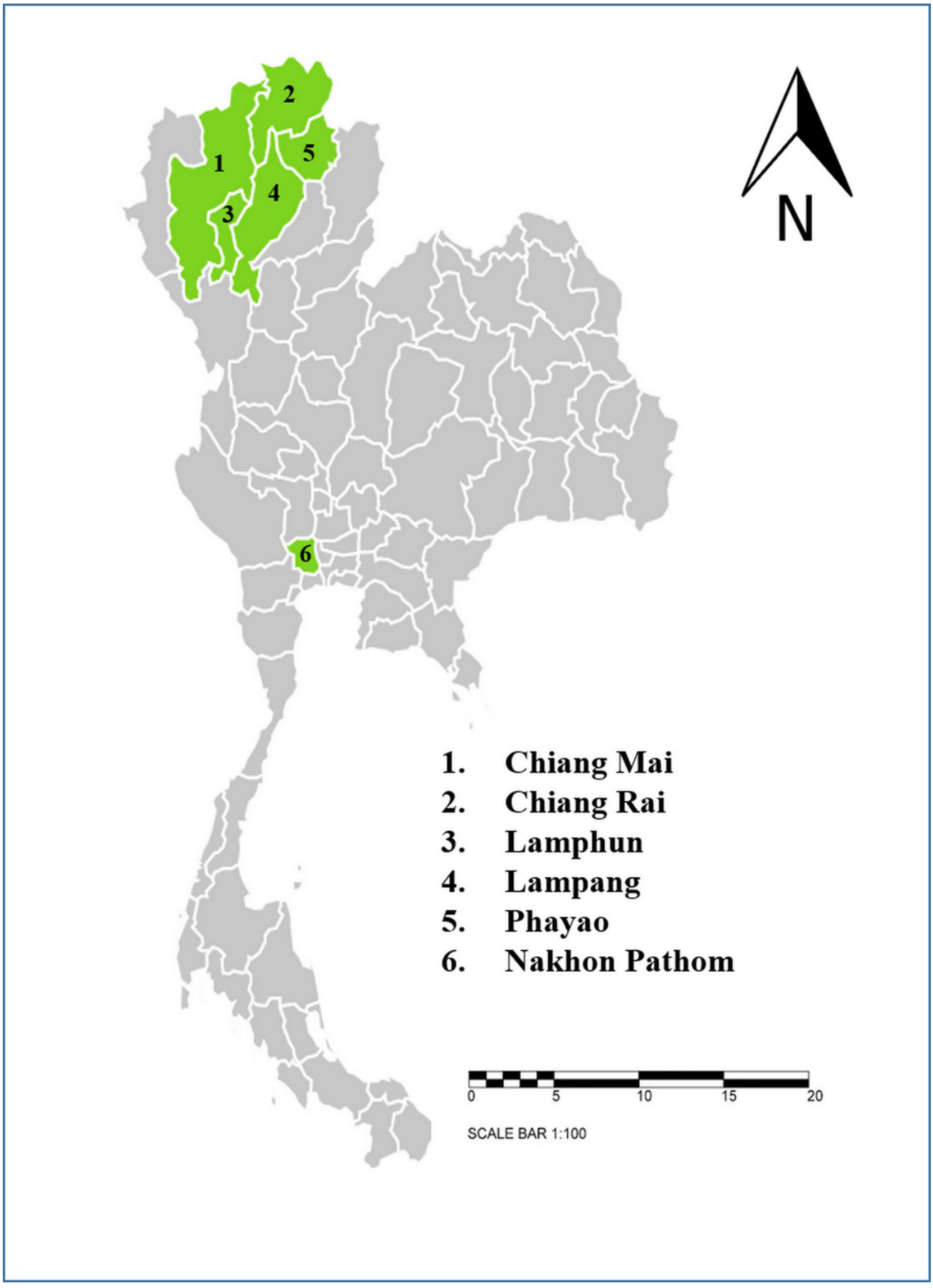
Table 3. Summary of PCR screening results for *B. bovis, T. orientalis*, and *A. marginale* single infections in cattle from the northern and central Thailand.

Province	No. of cattle	*B. bovis*		*T. orientalis*		*A. marginale*	
		Positive	%	Positive	%	Positive	%
Chiang Mai	143	0	0	71	49.65	5	3.50
Chiang Rai	87	1	1.15	2	2.30	2	2.30
Lamphun	557	7	1.26	200	35.90	187	33.57
Lampang	76	4	5.26	16	21.05	65	85.53
Phayao	122	0	0	92	75.41	92	75.41
Nakhon Pathom	81	1	1.23	8	9.87	13	16.05
Total	**1,066**	**13**	**1.22**	**389**	**36.50**	**364**	**34.15**

Figure 1

Sampling areas map in the northern and central of Thailand.

A total of 1,066 blood samples were collected from 6 provinces; 1. Chiang Mai (n=143), 2. Chiang Rai (n=87), 3. Lamphun (n=557), 4. Lampang (n=76), 5. Phayao (n=122) and 6. Nakhon Pathom (n=81). The map using an online infographic tool for map generation (https://create.piktochart.com).


2
1
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4
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6
N
1. Chiang Mai
2. Chiang Rai
3. Lamphun
4. Lampang
5. Phayao
6. Nakhon Pathom
0
5
10
15
20
SCALE BAR 1:100

Figure 2

Typical morphology of cattle hemoparasitic infection in a thin blood smear stained with 10% Giemsa showing multiple infected RBCs.

(A) *Babesia* spp.; (B) *Theileria* spp.; (C) *Anaplasma* spp.

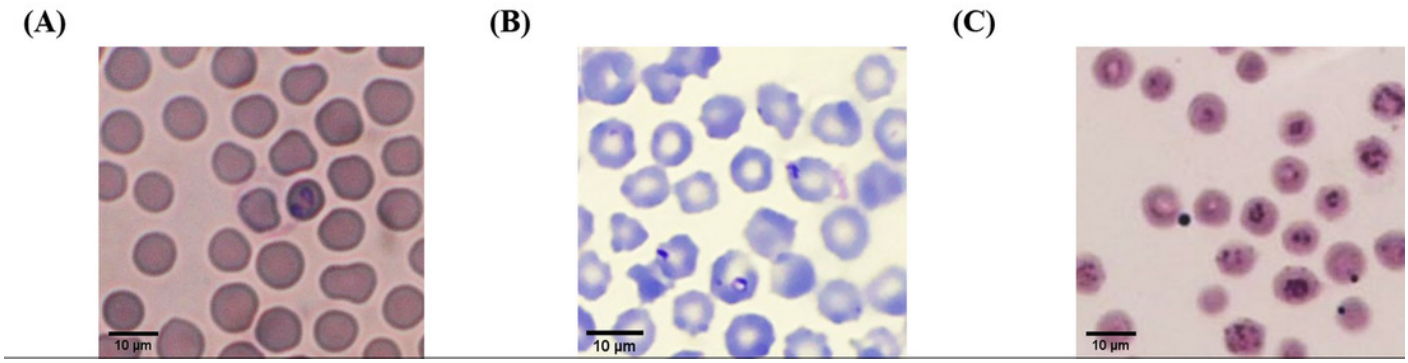

Figure 3

PCR detection of cattle hemoparasite infection

(A) *B. bovis* (298 bp) (B) *T. orientalis* (776 bp) (C) *A. marginale* (420 bp). The molecular size standard is a 100 bp ladder, positive and negative control DNA were also indicated.

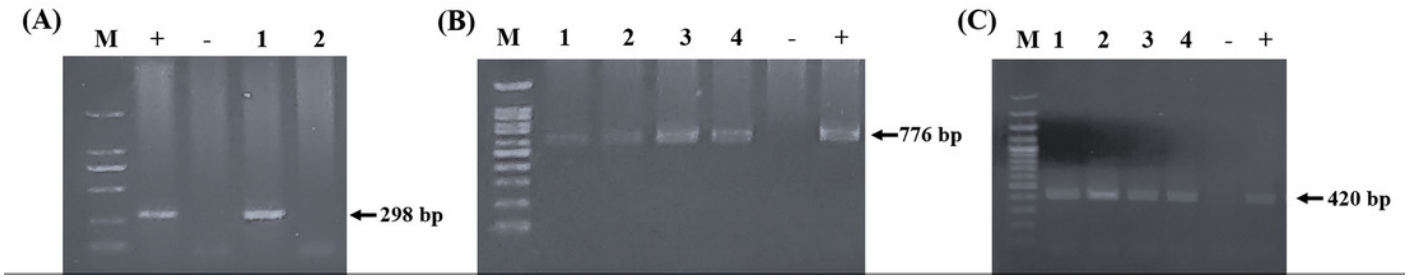

Figure 4

Venn diagram summarizing the species specificity and infection rate of cattle hemoparasite infections in the northern and central of Thailand.

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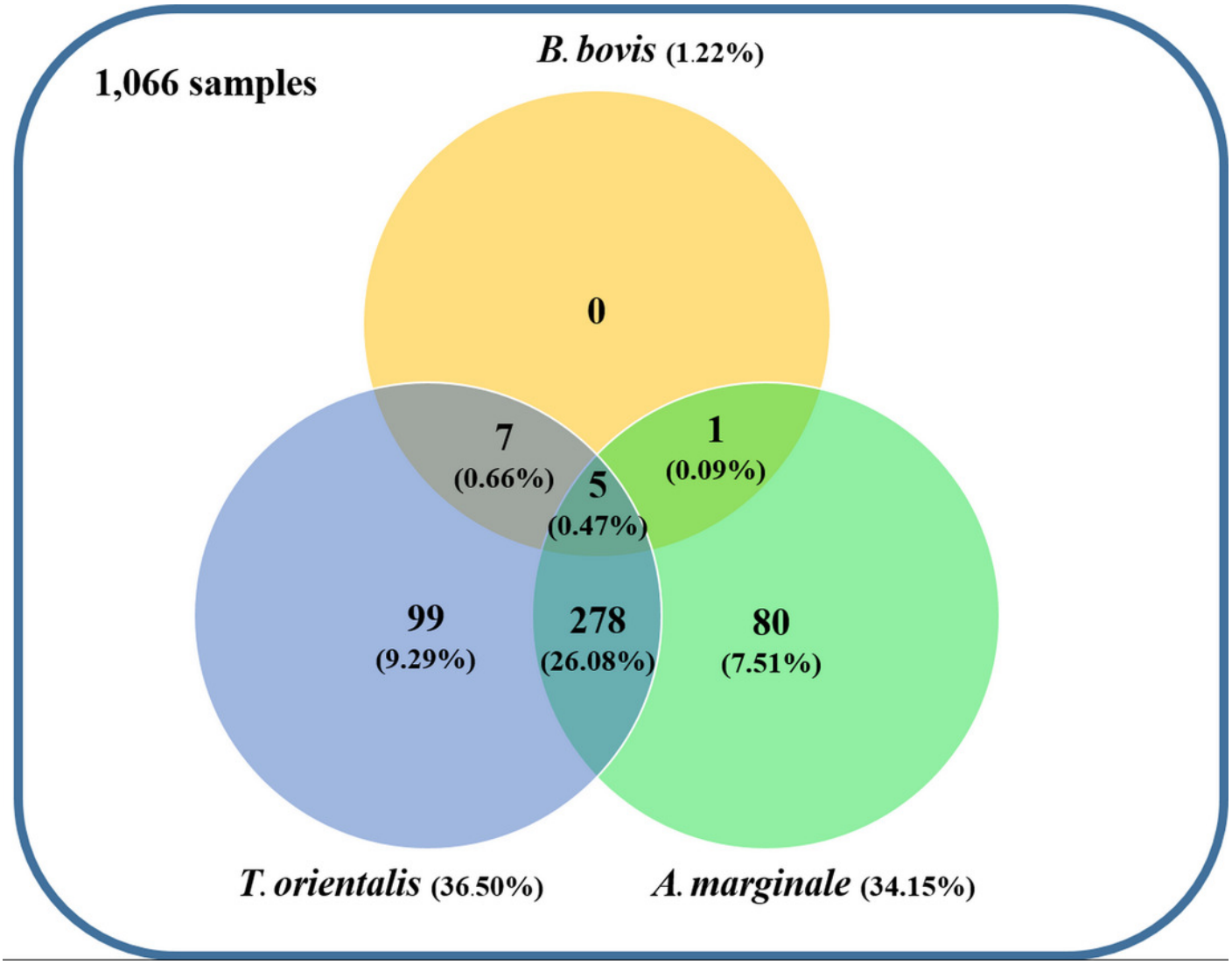

Figure 5

Phylogenetic relationships based on rap-1 sequence of B. bovis, in accordance with the PCR amplified sequence.

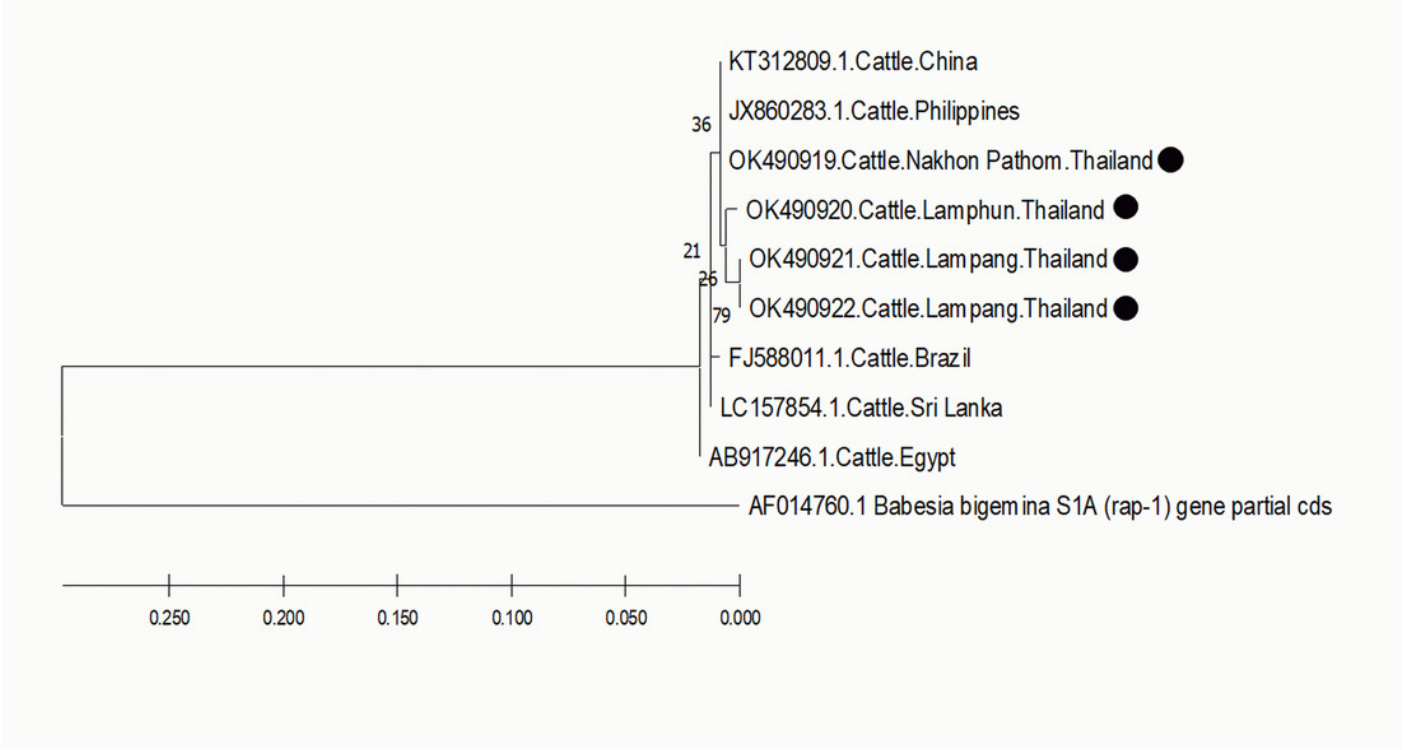

Figure 6

Phylogenetic relationships based on mpsp sequence of T. orientalis, in accordance with the PCR amplified sequence.

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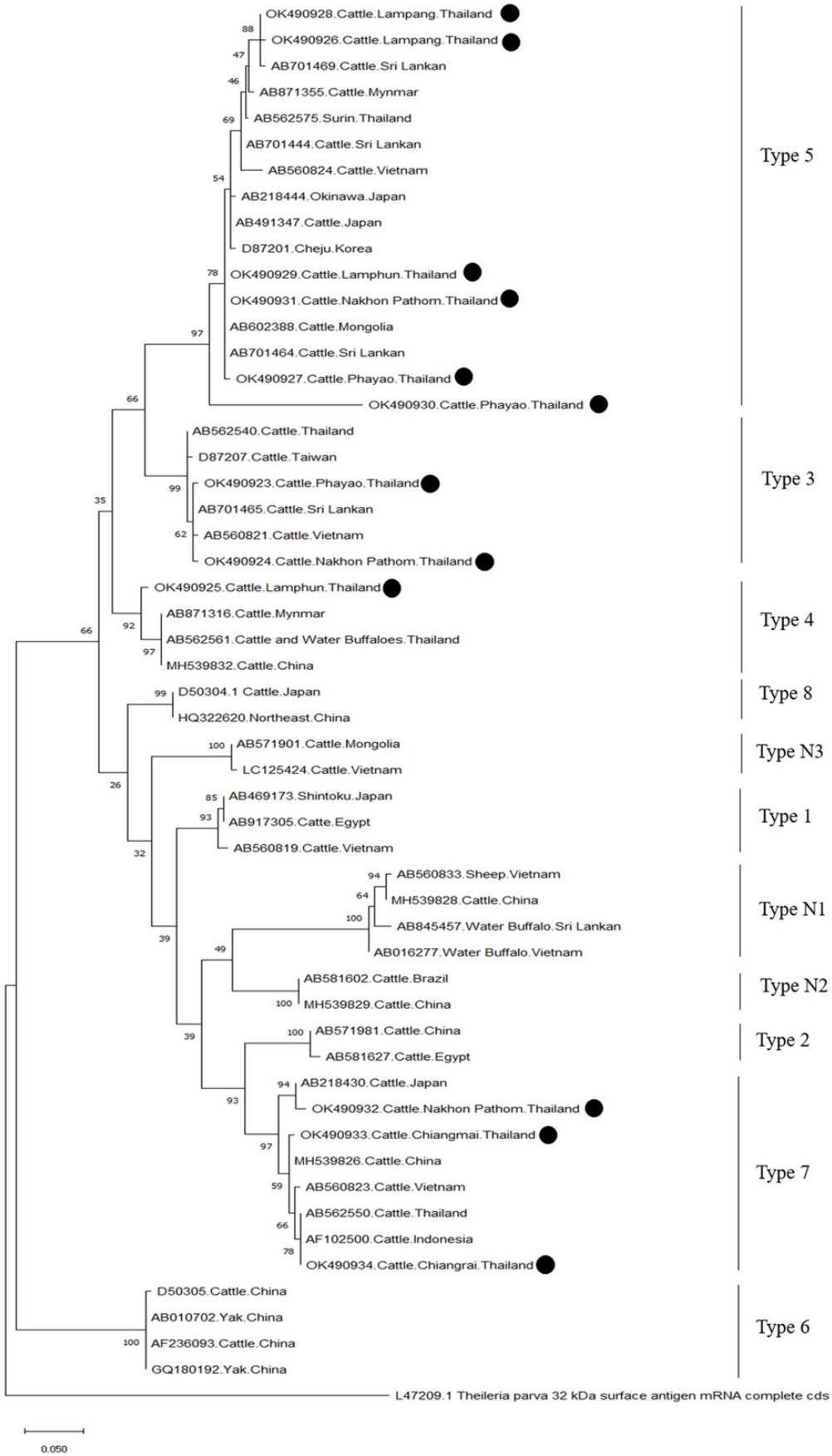
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OK490926.Cattle.Lampang.Thailand
AB701469.Cattle.Sri Lankan
AB871355.Cattle.Mynmar
AB562575.Surin.Thailand
AB701444.Cattle.Sri Lankan
AB560824.Cattle.Vietnam
AB218444.Okinawa.Japan
AB491347.Cattle.Japan
D87201.Cheju.Korea
OK490929.Cattle.Lamphun.Thailand
OK490931.Cattle.Nakhon Pathom.Thailand
AB602388.Cattle.Mongolia
AB701464.Cattle.Sri Lankan
OK490927.Cattle.Phayao.Thailand
OK490930.Cattle.Phayao.Thailand
AB562540.Cattle.Thailand
D87207.Cattle.Taiwan
OK490923.Cattle.Phayao.Thailand
AB701465.Cattle.Sri Lankan
AB560821.Cattle.Vietnam
OK490924.Cattle.Nakhon Pathom.Thailand
OK490925.Cattle.Lamphun.Thailand
AB871316.Cattle.Mynmar
AB562561.Cattle and Water Buffaloes.Thailand
MH539832.Cattle.China
D50304.1 Cattle.Japan
HQ322620.Northeast.China
AB571901.Cattle.Mongolia
LC125424.Cattle.Vietnam
AB469173.Shintoku.Japan
AB917305.Catte.Egypt
AB560819.Cattle.Vietnam
AB560833.Sheep.Vietnam
MH539828.Cattle.China
AB845457.Water Buffalo.Sri Lankan
AB016277.Water Buffalo.Vietnam
AB581602.Cattle.Brazil
MH539829.Cattle.China
AB571981.Cattle.China
AB581627.Cattle.Egypt
AB218430.Cattle.Japan
OK490932.Cattle.Nakhon Pathom.Thailand
OK490933.Cattle.Chiangmai.Thailand
MH539826.Cattle.China
AB560823.Cattle.Vietnam
AB562550.Cattle.Thailand
AF102500.Cattle.Indonesia
OK490934.Cattle.Chiangrai.Thailand
D50305.Cattle.China
AB010702.Yak.China
AF236093.Cattle.China
GQ180192.Yak.China
L47209.1 Theileria parva 32 kDa surface antigen mRNA complete cds
Type 5
Type 3
Type 4
Type 8
Type N3
Type 1
Type N1
Type N2
Type 2
Type 7
Type 6
0.050

Figure 7

Phylogenetic relationships based on msp4 sequence of A. marginale, in accordance with the PCR amplified sequence.

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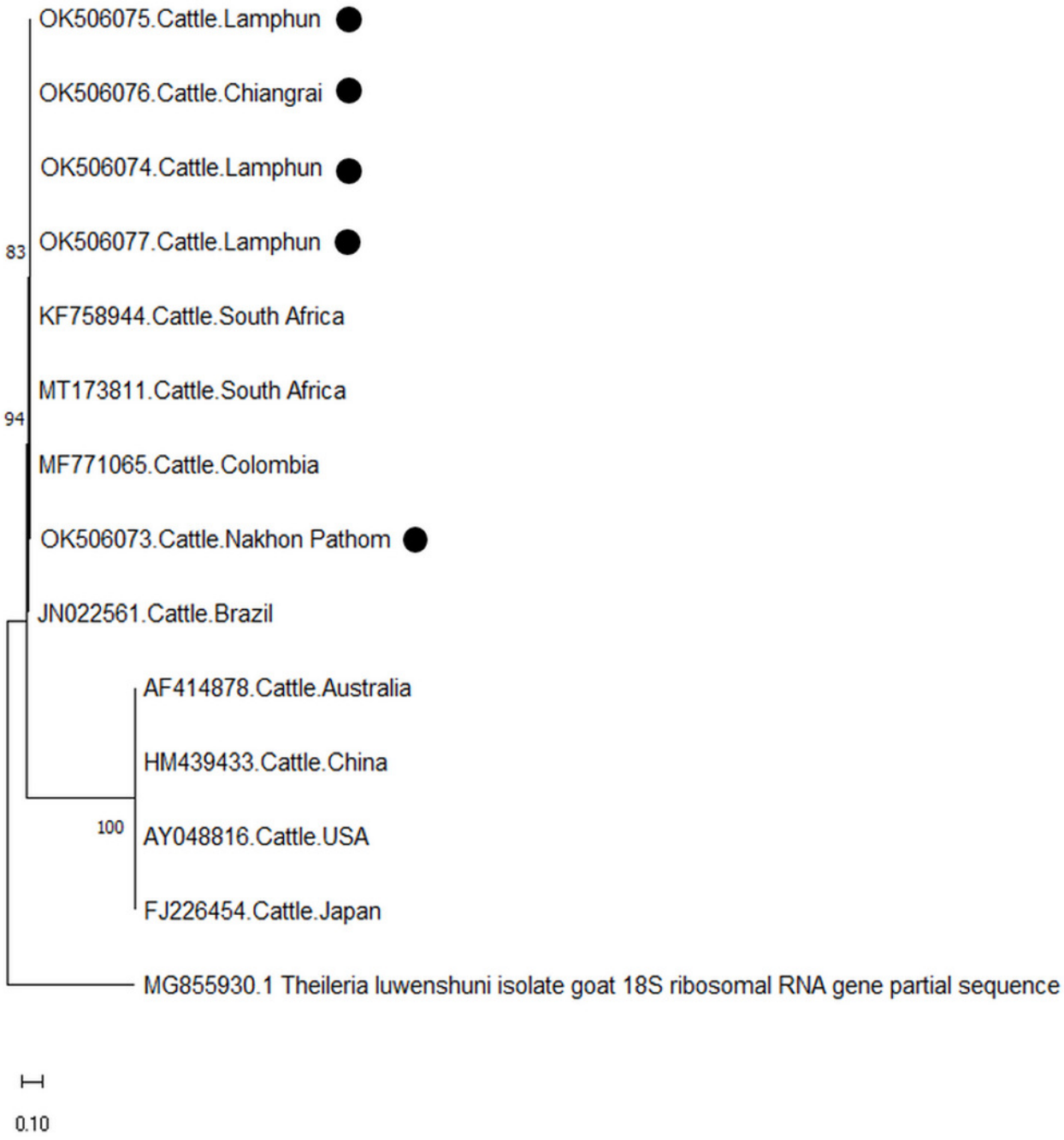
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OK506076.Cattle.Chiangrai
OK506074.Cattle.Lamphun
83
OK506077.Cattle.Lamphun
KF758944.Cattle.South Africa
MT173811.Cattle.South Africa
94
MF771065.Cattle.Colombia
OK506073.Cattle.Nakhon Pathom
JN022561.Cattle.Brazil
AF414878.Cattle.Australia
HM439433.Cattle.China
100
AY048816.Cattle.USA
FJ226454.Cattle.Japan
MG855930.1 Theileria luwenshuni isolate goat 18S ribosomal RNA gene partial sequence
0.10