Title

Molecular detection and genetic diversity of bovine *Babesia* spp., *Theileria orientalis* and *Anaplasma marginale* in beef cattle in Thailand

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22 **Abstract**

23 *Babesia* spp., *Theileria orientalis* and *Anaplasma maginale* are significant tick-borne
24 pathogens that affect the health and productivity of cattle in tropical and subtropical areas. In this
25 study we used PCR to detect the presence of *Babesia bovis*, *B. bigemina*, *T. orientalis* and *A.*
26 *marginale* in 608 beef cattle from 9 provinces of Thailand. The PCRs were performed using
27 species-specific primers based on the *B. bovis* spherical body protein 2 (BboSBP2), *B. bigemina*
28 rhoptry-associated protein 1a (BbiRAP-1a), *T. orientalis* major piroplasm surface protein
29 (ToMPSP) and *A. marginale* major surface protein 4 (AmMSP4) genes. To determine the genetic
30 diversity of the above parasites, amplicons of *B. bovis* and *B. bigemina* ITS1-5.8s rRNA gene-
31 ITS2 regions (*B. bovis* ITS, *B. bigemina* ITS), ToMPSP and AmMSP4 genes were sequenced for
32 phylogenetic analysis. The study revealed that *A. marginale* (14.5%) and *T. orientalis* (9.9%)
33 were the most prevalent single infections followed by *B. bigemina* (5.4%) and *B. bovis* (1.2%).
34 Co-infections of two or three parasites were observed in 37.7 % of the animals sampled.
35 Sequence analysis showed the BoSBP2 gene to be more conserved than *B. bovis* ITS in the
36 different isolates and similarly, the BiRAP-1a was more conserved than *B. bigemina* ITS. In the
37 phylogenetic analysis *T. orientalis* MPSP sequences were classified into type 3, 5 and 7 as
38 previously reported. *A. marginale* MSP4 gene sequences shared high identity and similarity with
39 each other and clustered with isolates from other countries. This study provides information on
40 the prevalence and genetic diversity of tick-borne pathogens in beef cattle and highlights the
41 need for effective strategies to control these pathogens in Thailand.

42 Keyword: Babesiosis, Benign theileriosis, Anaplasmosis, Phylogentic tree, Beef cattle, Thailand

43

44 **Introduction**

45 Tick-borne diseases (TBDs) such as babesiosis, theileriosis and anaplasmosis cause
46 economic losses to cattle production in many countries of the world (Uilenberg 1995). Bovine
47 babesiosis is caused by *Babesia bovis*, *B. bigemina*, *B. divergens*, *B. ovata* and *B. major* (Bock et
48 al. 2004). *B. bovis* and *B. bigemina* are generally considered the most economically important
49 pathogens in tropical and subtropical regions (Bock et al. 2004). The infection causes high fever,
50 anorexia and hemoglobinuria. However, *B. bovis* induces neurological damage and respiratory
51 symptoms in infected animals (Everitt et al. 1986).

52 Bovine theileriosis pathogens are classified into 2 types based on their ability to invade
53 leukocytes in infected cattle. *Theileria annulata* and *T. parva* are highly virulent
54 lymphoproliferative parasites that cause tropical theileriosis and east coast fever, respectively
55 (Mukhebi et al. 1992). Tropical theileriosis is commonly found in North Africa, South Europe
56 and Asia while east coast fever is mainly found in East, Central and South Africa (Weir et al.
57 2010). *Theileria orientalis* is a non-lymphoproliferative theileria parasite which is widely
58 distributed in Southeast Asia. The parasite is considered to be of low pathogenicity and causes
59 benign theileriosis, however, cases of clinical illness have been reported. Clinical findings
60 include weakness, icteric, pale mucous membranes, hemolytic anemia, diarrhea and poor milk
61 production (Izzo et al. 2010; Kamau et al. 2011). Severe disease is more frequently found in
62 naïve animals when they are introduced into endemic area (McFadden et al. 2011).

63 Anaplasmosis is an infectious disease caused by the rickettsial gram-negative pathogens
64 *Anaplasma marginale* and *A. centrale* (Aubry and Geale 2011). *A. marginale* is the most
65 prevalent tick-borne parasite of cattle worldwide (Kocan et al. 2010). Bovine anaplasmosis is
66 characterized by hemolytic anemia, high fever, weight loss, abortion and death (Richey and

67 Palmer 1990). Transmission of the pathogen in cattle occurs both biologically and mechanically
68 through ticks, blood sucking insects and blood contamination (Aubry and Geale 2011).
69 Noteworthy, *Babesia* spp. and *Anaplasma* spp. co-infections are often found in cattle (Altay et al.
70 2008; Suarez and Noh 2011).

71 The occurrence of causative agents of bovine babesiosis, benign theileriosis and bovine
72 anaplasmosis has been reported in different parts of Thailand (Tananyutthawongesea et al. 1999;
73 Altangerel et al. 2011b; Simking et al. 2013). However, most epidemiological studies on the
74 above pathogens focused on dairy cattle. This possibly explains the lack of information on their
75 prevalence in beef cattle in Thailand. The dominant beef cattle breed in Thailand is a native
76 breed. Due to their natural tolerance, native cattle that are infected with tick-borne pathogens
77 may show fewer clinical signs and act as reservoirs for infection of the herd (Jonsson et al. 2008).

78 Although a previous study has investigated the risk factors associated with *Babesia* spp.
79 and *T. orientalis* infections in beef cattle from North and Northeastern Thailand, (Jirapattharasate
80 et al. 2016), the prevalence of the above pathogens in other areas, the frequency of *A. marginale*
81 infection as well as the genetic diversity of these pathogens was not assessed. Therefore, the
82 purpose of this study was to investigate the prevalence of *Babesia* spp. and *T. orientalis* in beef
83 cattle from the Western part of Thailand. *A. marginale* prevalence in cattle from Northern,
84 Northeastern, and Western part of the country was also assessed. The genetic diversity of the
85 pathogens and their genetic relations between isolates from different countries were also
86 examined.

87

88 **Material and methods**

89 *Sample collection and DNA extraction*

90 A total of 608 blood samples were collected from randomly selected beef cattle in 9
91 provinces located in North, Northeastern and Western parts of Thailand between March 2014
92 and June 2015. The provinces surveyed were Chiangrai, Payao, Maehongson, Khonkan,
93 Mahasarakam, Loei, Kanchanaburi, Rachaburi and Nakhonpathom (Fig.1). The sample size was
94 calculated based on a method previously described (Humphry et al. 2004) using an online
95 epidemiological tool for sample size estimation (http://epitool.ausvet.com
96 au/content.php?page=PrevalenceSS). Blood samples were collected from the jugular or caudal
97 vein and immediately transferred into 10 ml vacuum blood collection tubes with EDTA-K_2. The
98 samples were placed on ice until arrival in the laboratory.

99 Genomic DNA was extracted and purified from 200 µl of blood using the QIAamp®
100 DNA blood Mini Kit (QIAGEN, Germany) according to the manufacturer's instruction. The
101 extracted DNA was stored at -30°C until it was used.

102

103 *Ethical statement*

104 The owners of the selected farms were informed of the study and provided their approval
105 for sampling of the cattle. Authorization for blood samples collection was provided by the
106 Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC), Mahidol
107 University Thailand. The protocol number is MUVS-2013-41.

108

109 *Molecular detection of Babesia spp., T. orientalis and A. marginale*

110 Previously described PCR primers were used to screen the cattle DNA samples for *B.*
111 *bovis* spherical body protein 2 (BoSBP2), *B. bigemina* rhoptry associated protein-1a (BiRAP-1a),
112 *T. orientalis* major piroplasm surface protein (ToMPSP) and *A. marginale* major surface protein
113 4 (AmMSP4) (AbouLaila et al. 2010; Terkawi et al. 2011; Ota et al. 2009; Torina et al. 2012).
114 Nested PCRs (nPCR) were carried out for *B. bovis* and *B. bigemina* infections, while single
115 PCRs were used to detect *T. orientalis* and *A. marginale* infections. The composition of the
116 reaction mixture and cycling condition of the PCR assays were performed according to
117 referenced studies (AbouLaila et al. 2010; Terkawi et al. 2011; Ota et al. 2009; Torina et al.
118 2012). The PCR products were subjected to electrophoresis on 1.5% agarose gel in 1x TAE
119 buffer, stained with ethidium bromide and visualized under UV transilluminator.

120

121 *PCR amplification of ITS1-5.8s rRNA gene-ITS2 genomic region of B. bovis and B. bigemina*

122 Genetic characterization of *Babesia* isolates was performed using ITS1-5.8s rRNA gene-
123 ITS2 regions (*B. bovis* ITS, *B. bigemina* ITS) as marker. Specific primers for nPCR were used
124 for the amplification as described previously (Cao et al. 2012). The reaction mixtures were
125 performed in a final volume of 20 µl, containing 2 µl of DNA sample, 0.25 mM of dNTPs, 1x of
126 PCR buffer, 1 pmol of each primer (Sigma-Aldrich, Japan), 0.1 U of *Taq*-polymerase (Ex-Taq
127 DNA polymerase, Takara, Japan). The thermocycling conditions consisted of denaturation for 5
128 min at 94ºC, 35 cycles of a second denaturation step at 94ºC (1 min) annealing step at 53ºC (1
129 min) for both *B. bovis* and *B. bigemina*, and extension at 72ºC (1 min), then a final extension at
130 72ºC for 10 min. The nPCR conditions were similar to the first PCR conditions, except that the
131 annealing temperature of *B. bigemina* was 55ºC.

132

133 *Cloning and sequencing*

134 For each parasite, six PCR products were randomly selected from among the positive
135 samples for cloning and sequencing. PCR products were extracted from agarose gel using
136 QIAquick Gel extraction kit (Qiagen, Germany), subsequently ligated into a pGEM-T Easy
137 Vector (Promega, USA) and transformed into *Escherichia coli* DH5α-competent cells. For each
138 sample, 2 selected colonies were expanded in overnight cultures and DNA was extracted using
139 Nucleospin® Plasmid QuickPure (MACHERY-NAGEL, Germany). The inserts of BoSBP2,
140 BiRAP-1a, ToMPSP, AmMSP4 genes, *B. bovis* and *B. bigemina* ITS regions in the purified
141 plasmids were sequenced using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems,
142 USA) and an ABI Prism 3100 140 genetic analyzer (Applied Biosystems, USA). The sequences
143 were analyzed using Bioedit version 7.2.5 (Tom Hall Ibis Biosciences, USA) and their identities
144 and similarities were determined by GenBank BLASTn analysis. The percent identities between
145 nucleotide sequences were computed by Pairwise distance using MEGA version 6.0 program
146 (Tamura et al.2013)

147

148 *Phylogenetic analysis*

149 Phylogenetic trees were constructed from *B. bovis* and *B. bigemina* ITS regions, ToMPSP
150 and AmMSP4 sequences obtained in this study along with corresponding sequences from other
151 regions of the world banked in genetic databases. Multiple sequence alignment was performed
152 for each locus with the ClustalW algorithm and then genetic relatedness was analyzed by
153 neighbor-joining phylogenetic tree using MEGA version 6.0 program (Tamura et al. 2013).

154 Bootstrap test with 1000 replications was used to estimate the confidence of the branching
155 pattern of the trees.
156

157 *Statistical analysis*

158 Confidence intervals (CI) were calculated for the prevalence of each pathogen using
159 online OpenEpi program (http://www.openepi.com/Proportion/Proportion.htm).
160

161 **Results**

162 *Prevalence of Babesia* spp.*, T. orientalis and A. marginale infections in cattle blood samples*

163 The prevalence of the bovine tick-borne pathogens determined in the present study is
164 shown in Table 1. The PCR assay revealed that *A. marginale* (14.5%) and *T. orientalis* (9.9%)
165 were the most prevalent single infections followed by *B. bigemina* (5.4%) and *B. bovis* (1.2%).
166 Multiple infections of two or more pathogens were apparent in 37.7 % (229/608) of the animals
167 sampled. *A. marginale* (82.1%, 188/229) was the pathogen most frequently associated with
168 multiple infections. Co-infections with *T. orientalis* (60.7%, 139/229) or *B. bigemina* (59.8%,
169 137/229) were observed in almost equal frequencies while *B. bovis* was found in 35.4% (81/229)
170 of the multiple infections (Table 2).
171

172 *Sequence analysis of BoSBP2, BiRAP-1a, ToMPSP, AmMSP4 genes, B. bovis and B. bigemina*
173 *ITS regions.*

174 *B. bovis* (n= 6) and *B. bigemina* (n=6) positive samples were used to analyze the partial
175 sequence of BoSBP2 (580 bp) and BiRAP-1a genes (412 bp). The nucleotide identity of
176 BoSBP2 sequences (KU764505-KU764510) was 90.1-99.8 % and they shared 90.2-97.9 %
177 identity with published sequences (AB742544, AB742545, AB742547, AB772320 and
178 AB772322). BiRAP-1a sequences in this study (KU764511-KU764516) were highly conserved
179 (99.8-100%) and shared 99.7-100 % identity with published sequences (AB617643, AB586126,
180 AB594817, and JX648554). Six *T. orientalis* MPSP gene sequences (776 bp) were obtained in
181 this study and shared 73.2-98.8% nucleotide identity with each other (KU764499-KU764504).
182 The nucleotide sequence identity value of *A. marginale* MSP4 (344 bp) (KU764493-KU764498)
183 was 99.1-100 % among obtained sequences and 97.4-100% with previously published sequences
184 (JN572928, AY665999, EU283844 and HM 640938).

185 The ITS regions were amplified from *B. bovis* and *B. bigemina* PCR positive samples to
186 further investigate their identity and their phylogenetic relationship. Six different sequences of *B.*
187 *bovis* ITS (KU841554-KU841559) were obtained and their length ranged from 520 to 544 bp
188 (Fig. 2). The identity among these sequences was 93.1-97.1% and they shared 92.7-96.7%
189 identity with sequences available in the GenBank (JN974304, EF547925, EF458291, EF458292
190 and EF458287). Furthermore, the six partial sequences of *B. bigemina* ITS (KU841548-
191 KU841553) were 97.3-99.6% identical to each other and shared 95.7-99.4% identities with
192 database sequences (JN974295, EF458262, EF458267, EF458249 and HM538263).

193

194 *Phylogenetic analysis of B. bovis and B. bigemina* ITS regions*, ToMPSP, and AmMSP4*
195 *sequences.*

196 The *B. bovis* ITS sequences were all found in the same clade (clade 3) together with
197 sequences from China (EF457925), Thailand (JN974304) and Brazil (EF458291, EF458287 and
198 EF458292) (Fig. 3). *B. bigemina* ITS phylogenetic tree (Fig. 4) showed that the sequences from
199 this study were scattered in different subgroups of the same clade (clade 1). Two isolates were
200 located in the same clade with previously reported sequences from China (HM538247,
201 HM538227 and HM538263). Three isolates were confined to the same clade as previously
202 published isolates from Thailand (JN 974295 and JN974296). Furthermore, one isolate showed a
203 close relationship with a sequence from Brazil (EF458243).

204 Phylogenetic analysis revealed that ToMPSP gene sequences in this study were classified
205 into 3 clades; type 3, type 5 and type 7 (Fig. 5). Two ToMPSP gene sequences were located in
206 type 3 clade, closely related to isolates reported in cattle from Mongolia (AB571893), Thailand
207 (AB562279) and Brazil (AB581622). The ToMPSP type 5 sequences were similar to China
208 (AB571967), Thailand (KT460099) and Japan (AB491347) sequences. In addition, ToMPSP
209 type 7 sequences were closely related to the type 7 sequence from Thailand (KT460098 and
210 AB562581), Japan (AB218430) and Vietnam (AB560823). AmMSP4 phylogenetic tree showed
211 6 clades (Fig. 6). The six isolates of AmMSP4 gene obtained, clustered under clade 1 and
212 showed close relationships with sequences from Australia (AY665997 and AY665999) and
213 China (JN572928 and HM640938).

214

215 **Discussion**

216 A previous epidemiological study of *Babesia* spp. and *T. orientalis* in beef cattle was
217 carried out in the North and Northeastern part of Thailand (Jirapattharasate et al. 2016). However,

218 the prevalence of the above parasites in Western Thailand and genetic characterization of the
219 causative agents have not been elucidated. In this study, we performed a molecular detection for
220 *Babesia* spp., *T. orientalis* and *A. marginale* in blood samples of beef cattle from 3 regions of
221 Thailand. Our findings showed that overall, 68.6% of the beef cattle sampled were infected with
222 *B. bovis*, *B. bigemina*, *T. orientalis* or *A. marginale*. The highest prevalence of the tick-borne
223 parasites was found in the Northern region which was followed by the Northeastern and Western
224 regions. The prevalence of the parasites in cattle from the Western region was significantly lower
225 than those from Northern and Northeastern regions (p <0.001).

226 *A. marginale* (14.5%) was more prevalent than other single infections. The infection rate
227 of *A. marginale* in this study is consistent with previous epidemiological studies in Thailand that
228 showed 14.3-23.2% prevalence (Saetiew et al. 2014, 2015). The high prevalence of *A. marginale*
229 could be attributed to the various modes of transmissions of the parasites. Transmission of *A.*
230 *marginale* to cattle occurs biologically through ticks and biting files as well as in-utero through
231 the transplacental route (Aubry and Geale 2010). In Thailand the prevalence of *A. marginale*
232 depends on many different factors such as age of animal, tick distribution and season (Saetiew et
233 al. 2014).

234 Among tick-borne protozoan infections, the prevalence of *T. orientalis* (9.9%) was higher
235 than *B. bigemina* (5.4%) and *B. bovis* (1.2%). The high infection rate of *T. orientalis* in this study
236 is similar to a previous study that showed high prevalence of *T. orientalis* in beef cattle from
237 North and Northeastern, Thailand (Jirapattharasate et al. 2016).

238 *Haemaphysalis longicornis* and other *Haemaphysalis* spp. are reported as the main vector
239 for *T. orientalis* (Fujisaki 1992). These ticks however are not found on Thai cattle, which are
240 mainly infested with *Rhipicephalus microplus* (Changbunjong et al. 2009). Although previous

241 researches in Vietnam (Altangerel et al. 2011a) and China (Chen et al. 2014) detected *T.*
242 *orientalis* in engorged female *R. microplus*, all transovarial and trans-stadial attempts to transmit
243 the pathogens with that tick have been unsuccessful (Riek, 1982). Recently, Hammer et al (2016)
244 reported that *T. orientalis* can also be transmitted by biting arthropods (*Linognathus vituli*) and
245 colostral transfer. Further studies covering both ticks and biting arthropods are of interest to
246 elucidate the modes of transmission of *T. orientalis* in Thailand.

247 The high prevalence of *B. bigemina* compared to *B. bovis* in this study also agrees with
248 previous reports from the Philippines (Ybañez et al. 2013). According to Oliveira-Sequeira et al.
249 (2005) female *R. microplus* ticks in Brazil are more frequently infected with *B. bigemina* than *B.*
250 *bovis*. Hence, tick vector distribution and tick-*Babesia* spp. relationships may explain our results.
251 However, Thailand has a different ecosystem than Brazil and therefore further studies on
252 *Babesia* spp. prevalence in tick vectors in Thailand are required to clarify our findings.

253 This study also found mixed infections in 229 (37.7%) of the beef cattle samples. Co-
254 infection of *A. marginale* and *T. orientalis* (9.7%) was higher than co-infections with *A.*
255 *marginale* and *B. bigemina* (4.8%) as well as *B. bigemina* and *T. orientalis* (4.6%). Furthermore,
256 *A. marginale- B. bigemina-T. orientalis* infection was found in 5.8% of samples. Cattle that are
257 infected with multiple TBDs show more clinical signs and hematological abnormalities than
258 those infected with a single parasite (Hofmann-Lehmann et al. 2004). Hence, we recommend
259 further comparative studies on the pathology of both single and co-infections as well as host-
260 parasite interactions in different breeds of cattle in Thailand.

261 The sequence of *B. bovis* SBP2 was conserved among the cattle samples with 90.1-
262 99.8 % identity and shared 90.2-97.9 % similarity with sequences published in the GenBank
263 database. In addition, *B. bigemina* RAP-1a sequences in this study also showed high identities

264 with each other (99.8-100%) and homology with other geographic isolates (99.7-100 %). These
265 results confirmed that the two genes are highly conserved among geographic isolates and
266 valuable targets for detection of parasites from different areas (AboLaila et al. 2010; Terkawi et
267 al. 2011; Ibrahim et al. 2013; Nagano et al. 2013; Simking et al. 2013).

268 In this study we also identified and analyzed *B. bovis* and *B. bigemina* ITS regions in beef
269 cattle. Previous studies have reported that these genetic regions are useful for identification of
270 new species as well as differentiation between parasite species and subspecies (Bostrom et al.
271 2008; Niu et al. 2009). The sequences of the *B. bovis* ITS region were more diverse in their
272 nucleotide length and nucleotide identity (93.1-97.1%) than *B. bigemina* ITS sequences. These
273 results were in agreement with a previous study on *Babesia* spp. isolated from cattle in Thailand
274 which demonstrated that the sequences of *B. bovis* ITS have higher nucleotide variability than *B.*
275 *bigemina* ITS (Cao et al. 2012). However, in the phylogenetic tree, *B. bovis* ITS formed a
276 monophyletic clade with other known *B. bovis* ITS sequences (Fig. 3). Furthermore, *B. bigemina*
277 ITS also clustered in one clade (Fig. 4). This finding suggests that *Babesia* spp. isolates might
278 belong to the same species but that different strains of *B. bovis* and *B. bigemina* exist (Cao et al.
279 2012; Zhou et al. 2016).

280 The MPSP gene has been recognized as an epidemiological molecular marker for
281 identification and characterization of the genetic diversity of *T. orientalis* (Altangerel et al.
282 2011b; Sivakumar et al. 2014). Recently, *T. orientalis* isolates from different countries have been
283 divided into 11 genotypes (Type1-8 and type N1-N3) based on their MPSP gene sequences
284 (Sivakumar et al. 2014). In the present study the MPSP sequences obtained in the Western part
285 were compared to sequences previously reported in the Northern and Northeastern parts of
286 Thailand (Jirapattharasate et al. 2016). Previous molecular characterization of *T. orientalis* in

287 cattle in Thailand classified MPSP gene isolates into 5 genotypes (type 1,3,5,7 and N3)
288 (Altangerel et al. 2011b). However, phylogenetic analysis in this study revealed that *T. orientalis*
289 MPSP sequences were classified into 3 types (type 3, 5 and 7). The current study was unable to
290 detect MPSP genotype 1 and N3 probably due to the limited number of samples, farms, or area
291 covered. Therefore, a large-scale study with an increased number of samples from different
292 provinces needs to be undertaken.

293 The MSP4 gene was considered as a stable marker for genetic characterization of
294 *Anaplasma* spp. (De la Fuente et al. 2005). The sequences of the MSP4 gene obtained in this
295 study were highly conserved among the cattle isolates as well as the *A. marginale* MSP4
296 sequences from previous reports (Liu et al. 2012; Zhou et al. 2016). Phylogenetic analysis
297 revealed that all the MSP4 sequences were clustered into one clade (clade 1) with sequences
298 from Australia, China and Mexico. These results suggest that *A. marginale* isolates from cattle in
299 this study belong to one genotype. Previous studies correlated the genetic diversity of *A.*
300 *marginale* to animal movement (Palmer et al. 2001; De la Fuente et al. 2005). Therefore,
301 restricted movement of beef cattle might explain the absence of genetic diversity of *A. marginale*
302 in this study.

303 In conclusion, the results of the present study showed that *Babesia* spp., *T. orientalis* and
304 *A. marginale* are prevalent among beef cattle of the North, Northeastern and Western regions of
305 Thailand. In the genetic characterization, different *B. bovis,* and *B. bigemina* isolates, three *T.*
306 *orientalis* MPSP genotypes and one *A. marginale* MSP4 genotype were identified. These
307 findings improve the understanding of the epidemiology of tick borne pathogens in Thailand and
308 will certainly contribute to the devise of effective control strategies.

309

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457 *Anaplasma marginale* in Turkey. Ticks Tick Borne Dis 7: 126-134
458
459
460
461 **Figure captions**

462 **Fig. 1** Map of Thailand indicating areas where samples were collected. Areas investigated during
463 previous survey (North and Northeastern provinces) are indicated in green color, and provinces
464 added in the current study (Western provinces) are in yellow color. Study provinces: 1.) Chiang

465 rai, 2.) Payao, 3.) Mae hong sorn, 4.) Khonkan, 5.) Mahasarakham, 6.) Loei 7.) Kanchanaburi,
466 8.) Rachaburi and 9.) Nakhonpathom.

467 **Fig. 2** Nucleotide alignment of ITS1-5.8s rRNA gene-ITS2 sequences from 6 *B. bovis* isolates
468 from beef cattle.

469 **Fig. 3** Phylogenetic tree based on *B. bovis* ITS1-5.8s rRNA gene-ITS2 region sequences from
470 this study (boldface letters) and sequences retrieved from the Genbank database. Genbank
471 Accession Numbers for all sequences are shown. Bootstrap values are provided at the beginning
472 of each branch. The ITS1-5.8s rRNA gene-ITS2 region sequence of *B. bigemina* (HM538271)
473 was used as outgroup.

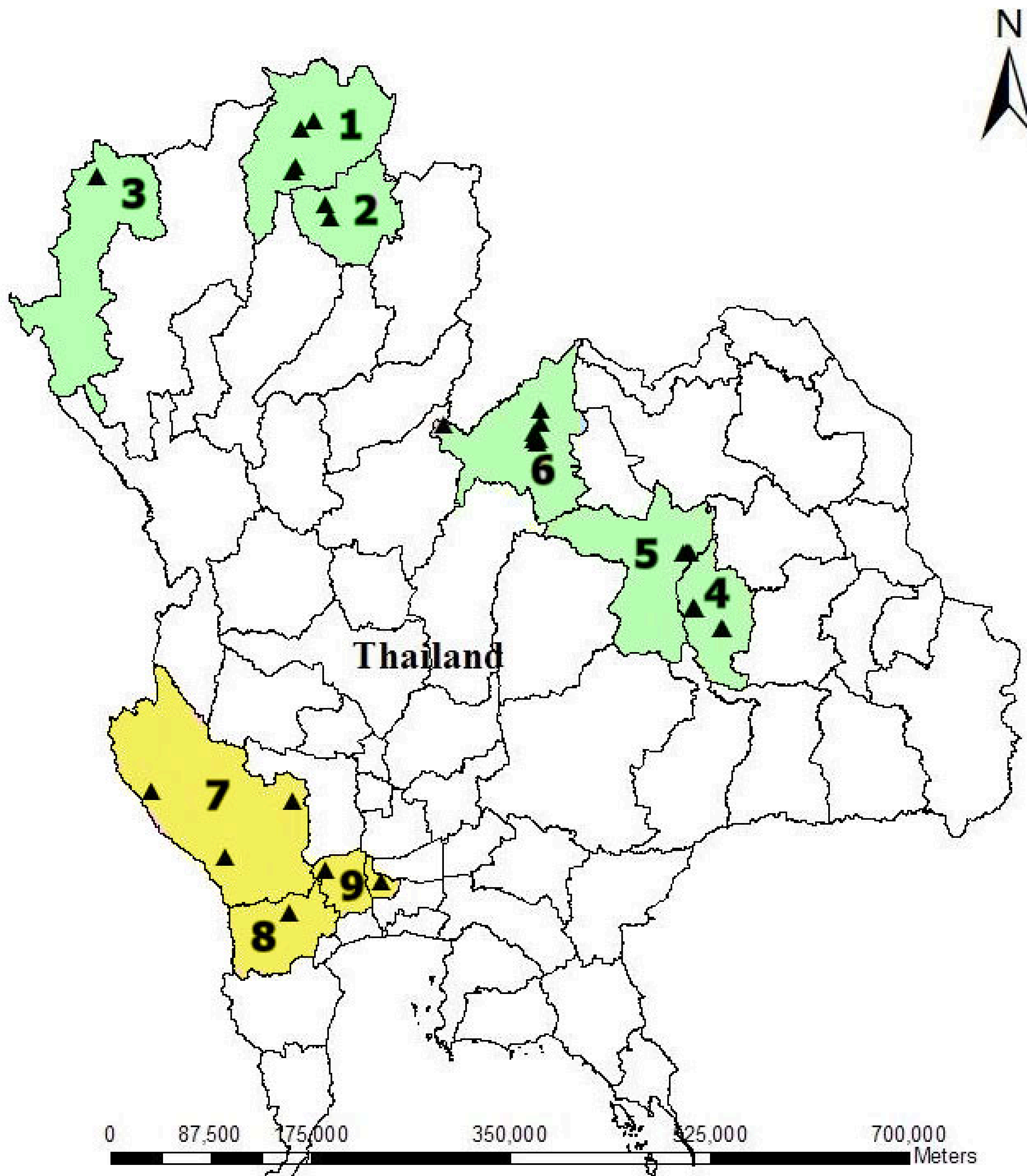
474 **Fig. 4** Phylogenetic tree based on *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region sequences
475 from this study (boldface letters) and sequences retrieved from the Genbank database. Genbank
476 Accession Numbers for all sequences are shown. Bootstrap values are provided at the beginning
477 of each branch. The ITS1-5.8s rRNA gene-ITS2 region sequence of *B. bovis* (HQ264131) was
478 used as outgroup.

479 **Fig. 5** Phylogenetic tree based on *T. orientalis* based on MPSP gene sequences from this study
480 (boldface letters) and sequences retrieved from the Genbank database. Genbank Accession
481 Numbers for all sequences are shown. Bootstrap values are provided at the beginning of each
482 branch. The Tams1 gene of *T. annulata* (JX683683) and *T. parva* (L47209) were used as
483 outgroup.

484 **Fig. 6** Phylogenetic tree based on *A. marginale* based on MSP4 gene sequences from this study
485 (boldface letters) and sequences retrieved from the Genbank database. Genbank Accession
486 Numbers for all sequences are shown. Bootstrap values are provided at the beginning of each

487 branch. The MSP4 gene sequence of *A. ovis* (HQ456347) and *A. centrale* (AF428090) were used

488 as outgroup.


N
1
2
3
4
5
6
7
8
9
Thailand
0
87,500
175,000
350,000
525,000
700,000
Meters

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                   10         20         30         40         50         60         70         80         90        100        110        120
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Sample 1   CACCACCAGT GGAAGCACAG CTTCCACGA- ----GTACTA CGTACTCGCG AGCACTCCGT GCTCACGGCA CCTCCGGTGC CACT-GATCG CCTT-TGGCG ATCTGGCAAC GCCGGCTACC
Sample 2   .......... .......... .......CCA ACGA...... .......... ......G... .......... .......... ....------ ---------- -A..A..... ..........
Sample 3   .......... .......... .......C-- ---------- ---------T. ......A... .......... .......... ....C..... ....-..... .......... ..........
Sample 4   .......... .......... .......CT- ---------- ----------. .......... .......... ...T...... ....------ ---------- -A..A..... ..........
Sample 5   .......... .......... ...T.....- ----...... .......... .......... .......... ...T...... ....C..... ....GC.... .........G. ..........
Sample 6   .......... .......... .......TC- --GA...... .......... .......... .......... ...T...... ....------ ---------- -A........ ..........

                  130        140        150        160        170        180        190        200        210        220        230        240
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Sample 1   CTAGTAGCCG GTTGGGGCTC CGCCCCCGTT GCTCCCCACC CCGAGGGCCG TGACTGCCAC GACCCGGGTT AAGCTCGCCT CGGCGAGATG CACCCC---T TTTGGGGGTG CCCTACTTTC
Sample 2   .......... .......... .......... .......... .......... .......... .......... .......... .......... ......CT-. .......... ..T.T.-.A.
Sample 3   .......... .......... .......... .......... .......... .......... .......... .........C .......... ......CCT. .......... ..-----.A.
Sample 4   .......... .C........ .......... .......... .......... .......... .......... .......... .......... ......CT-. .......... ..T.T.A...
Sample 5   .......... .......... .......... .......... .......... .......... .......... .........C .......... ......CCAA AA.....-.. TT----.A..
Sample 6   .......... .......... .A........ .......... .......... .......... .......... .......... .......... ......CT-. .......... ..T.T.-.A.

                  250        260        270        280        290        300        310        320        330        340        350        360
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Sample 1   CAGCCCTTCT TTAAGGGCTG GCACAACCAC T-CACACTTA TTA-CACTAC CTAAACTCCC AGCGATGGAT GCCTCGGCTC GCGCCTCGAT GAAGGACGCA GCAAAGTGCG ATATCCAGCA
Sample 2   ........T- ---....... .......... .CACT.GACC A.TA...... .......... .......... .......... .......... .......... .......... ..........
Sample 3   ........T- ---....... .......... A-GCAC.AC. CA.T...... .......... .......... .......... .......... .......... .......... ..........
Sample 4   .......... .......... .......... .CACT.GACC A.TA.....T T.C....... .......... .......... .......... .......... .......... ..........
Sample 5   .......... .......... ....C..T.. A-GCACAC.G ACTA....-- .......... .......... .......... .......... .......... .......... ..........
Sample 6   ........T- ---....... .......... .CACT.GACC A.TA...... .......... .......... .......... .......... .......... .......... ..........

                  370        380        390        400        410        420        430        440        450        460        470        480
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Sample 1   TGATTTGCAA CTTCTTGCGA TTGCTAGACC TCTGAACGTA ACCAACACAC T-CTTGTACG TCCATCTCAG TAAATTTCCA GTATGGTGTG ACACACCACC AGTGT--TGC ACCGCCTTGG
Sample 2   .......... .......... ........T. .......... .......... .TT....... .......... .G...CC... .......... .........T .....GT... .......C.A.
Sample 3   C......... .......... .......... .......... .......... .-TA...... .......... .G.....T.T A......... .........T .....GT... .......C..
Sample 4   .......... .......... .........T .......... .......... .TA.-..... .......... .G...CC.A. .......... .......... .....GT... .......C..
Sample 5   .......... .......... .......... .......... .......... .CT....... .......... .G....C..G .......... .......... .....GT... .......C..
Sample 6   .......... .......... .......... .......... .......... .CT.-..... .......... .G........ .......... .........T .....GT... ..........

                  490        500        510        520        530        540        550
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....
Sample 1   CGGTGCCTCT ACACCGCTCT TACTAGAGG- CAC-ACTGTG ACCCCGACAC GATAGATTTA TAGTGTCCAT GGGGCACAA
Sample 2   .......... .........C .T.AC....A ...T...... .......... ......A... .......... .........
Sample 3   .......... .......... .........- ...-...... .......... .......... .......... .........
Sample 4   ........A. .........C .T.AC....A ...T...... .......... ........C. G......... .........
Sample 5   .......... .........C .T.AC....- ...-...... .......... .......... G......... .........
Sample 6   .......... .....T.... ...-CAGA.G ...-...... .......... .......... .......... .........
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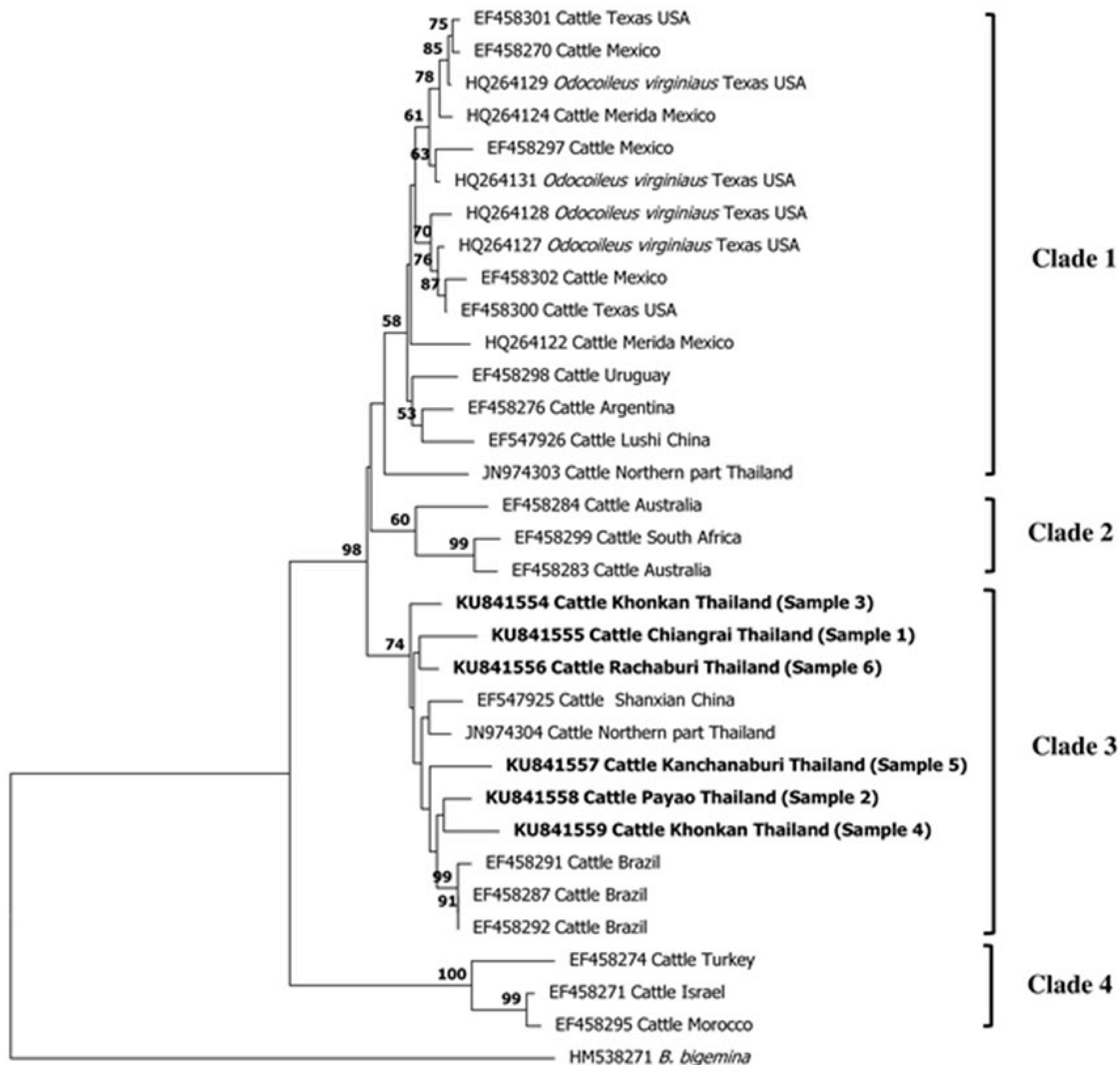

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EF458297 Cattle Mexico
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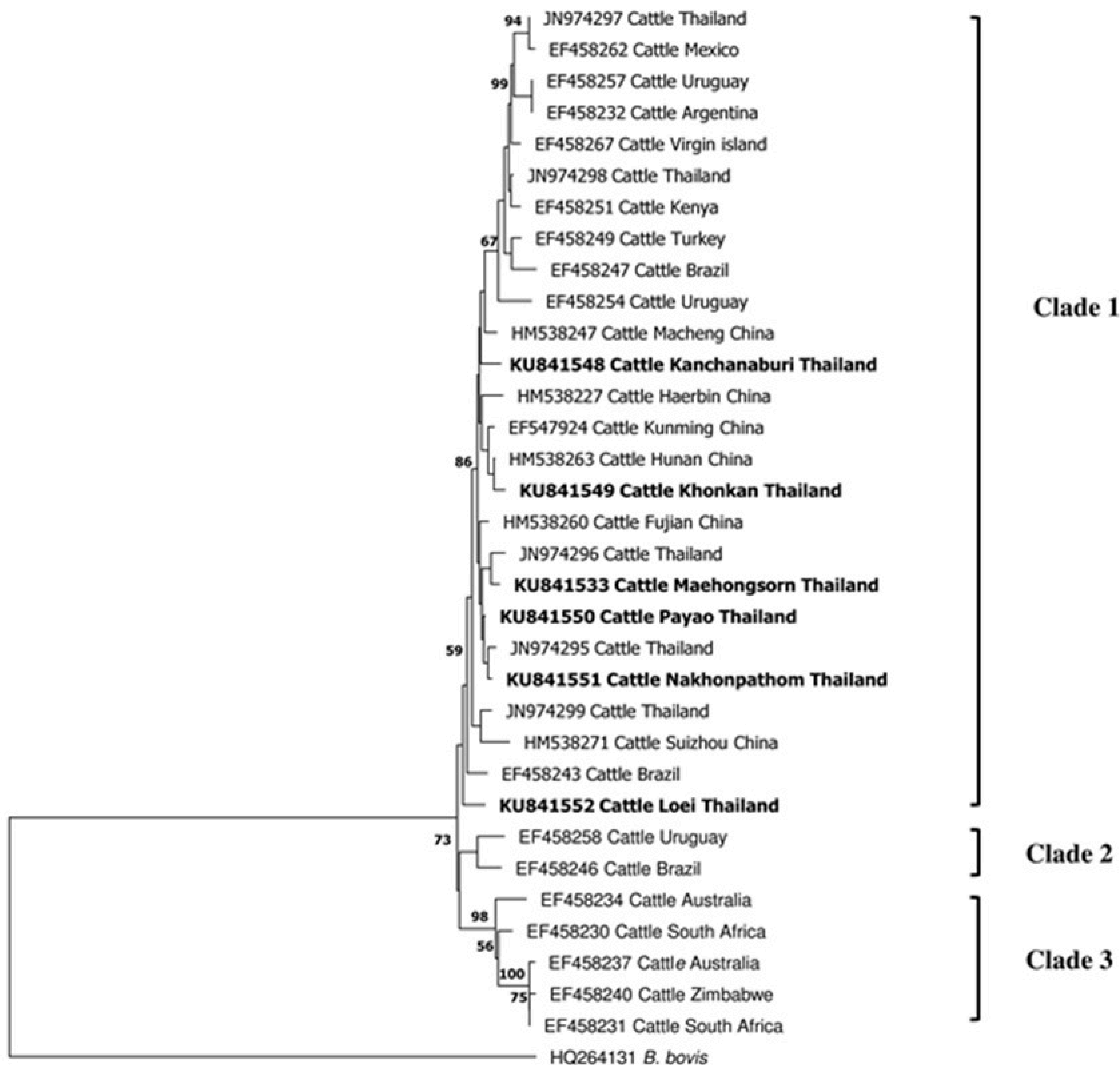
HQ264128 Odocoileus virginiaus Texas USA
HQ264127 Odocoileus virginiaus Texas USA
EF458302 Cattle Mexico
EF458300 Cattle Texas USA
HQ264122 Cattle Merida Mexico
EF458298 Cattle Uruguay
EF458276 Cattle Argentina
EF547926 Cattle Lushi China
JN974303 Cattle Northern part Thailand
EF458284 Cattle Australia
EF458299 Cattle South Africa
EF458283 Cattle Australia
KU841554 Cattle Khonkan Thailand (Sample 3)
KU841555 Cattle Chiangrai Thailand (Sample 1)
KU841556 Cattle Rachaburi Thailand (Sample 6)
EF547925 Cattle Shanxian China
JN974304 Cattle Northern part Thailand
KU841557 Cattle Kanchanaburi Thailand (Sample 5)
KU841558 Cattle Payao Thailand (Sample 2)
KU841559 Cattle Khonkan Thailand (Sample 4)
EF458291 Cattle Brazil
EF458287 Cattle Brazil
EF458292 Cattle Brazil
EF458274 Cattle Turkey
EF458271 Cattle Israel
EF458295 Cattle Morocco
HM538271 B. bigemina
Clade 1
Clade 2
Clade 3
Clade 4
75
85
78
61
63
70
76
87
58
53
60
99
98
74
99
91
100
99
0.02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EF458247 Cattle Brazil
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KU841533 Cattle Maehongsorn Thailand
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JN974299 Cattle Thailand
HM538271 Cattle Suizhou China
EF458243 Cattle Brazil
KU841552 Cattle Loei Thailand
EF458258 Cattle Uruguay
EF458246 Cattle Brazil
EF458234 Cattle Australia
EF458230 Cattle South Africa
EF458237 Cattle Australia
EF458240 Cattle Zimbabwe
EF458231 Cattle South Africa
HQ264131 B. bovis
94
99
67
86
59
73
98
56
100
75
Clade 1
Clade 2
Clade 3
0.02

AB560827 Cattle Hue Vietnam
KT460097 Cattle Northeastern Thailand
AB562552 Cattle Lampang Thailand
KT460099 Cattle Northern Thailand
KU764501 Cattle Chiangrai Thailand
AB571967 Cattle Fujian China
AB491347 Cattle Aso Japan
KU764499 Cattle Kanchanaburi Thailand
Type 5
EU584238 Cattle Hubei China
AB562541 Cattle Chiang Mai Thailand
AB562579 Water buffalo Roi Ed Thailand
AB581622 Cattle Bahia Brazil
KU764500 Cattle Nakornpathom Thailand
AB571893 Cattle Khentii Binder Mongolia
KU764503 Cattle Khonkan Thailand
Type 3
AF236095 Cattle Brisbane Australia
AB562561 Water buffalo Roi Ed Thailand
Type 4
D87191 Cattle Chonju Korea
D50304 Cattle Aomori Japan
HQ322620 Cattle Northeast China
Type 8
AB571901 Cattle Uvurkhangai Bogd Mongolia
AB562532 Cattle Chiang Rai Thailand
AB562560 Water buffalo Roi Ed Thailand
AB571884 Cattle Thua Thien Hue Vietnam
AB581603 Cattle Bahia Brazil
Type N3
AB560819 Cattle Hue Vietnam
AB571978.1 Cattle Fujian China
AB701473 Cattle Jaffna Sri Lanka
AB562544 Cattle Chaing Mai Thailand
AB562571 Water buffalo Burirum Thailand
Type 1
DQ078264 Jilin China
D87190 Chonju Korea
D11046 Cattle Ikeda Japan
Type 2
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AB218430 Cattle Okinawa Japan
KT460094 Cattle Northeastern Thailand
KU764504 Cattle Mahasarakam Thailand
KT460098 Cattle Northern Thailand
KU764502 Cattle Maehongsorn Thailand
AB562581 Water buffalo Surin Thailand
AB560823 Cattle Hue Vietnam
Type 7
AB845443 Water buffalo Polonnaruwa Sri Lanka
AB560829 Water Buffalo Thua Thien Hue Vietnam
AB562566 Water buffalo Roi Ed Thailand
AB581602 Cattle Bahia Brazil
Type N2
AB016277 Water buffalo Nha-Trang Vietnam
AB560833 Sheep Thua Thien Hue Vietnam
AB845479 Water buffalo Mannar Sri Lanka
AB845457 Water buffalo Polonnaruwa Sri Lanka
Type N1
AB010703 Cattle Kamphaeng Saen Thailand
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L47209 Tpms1 T. parva
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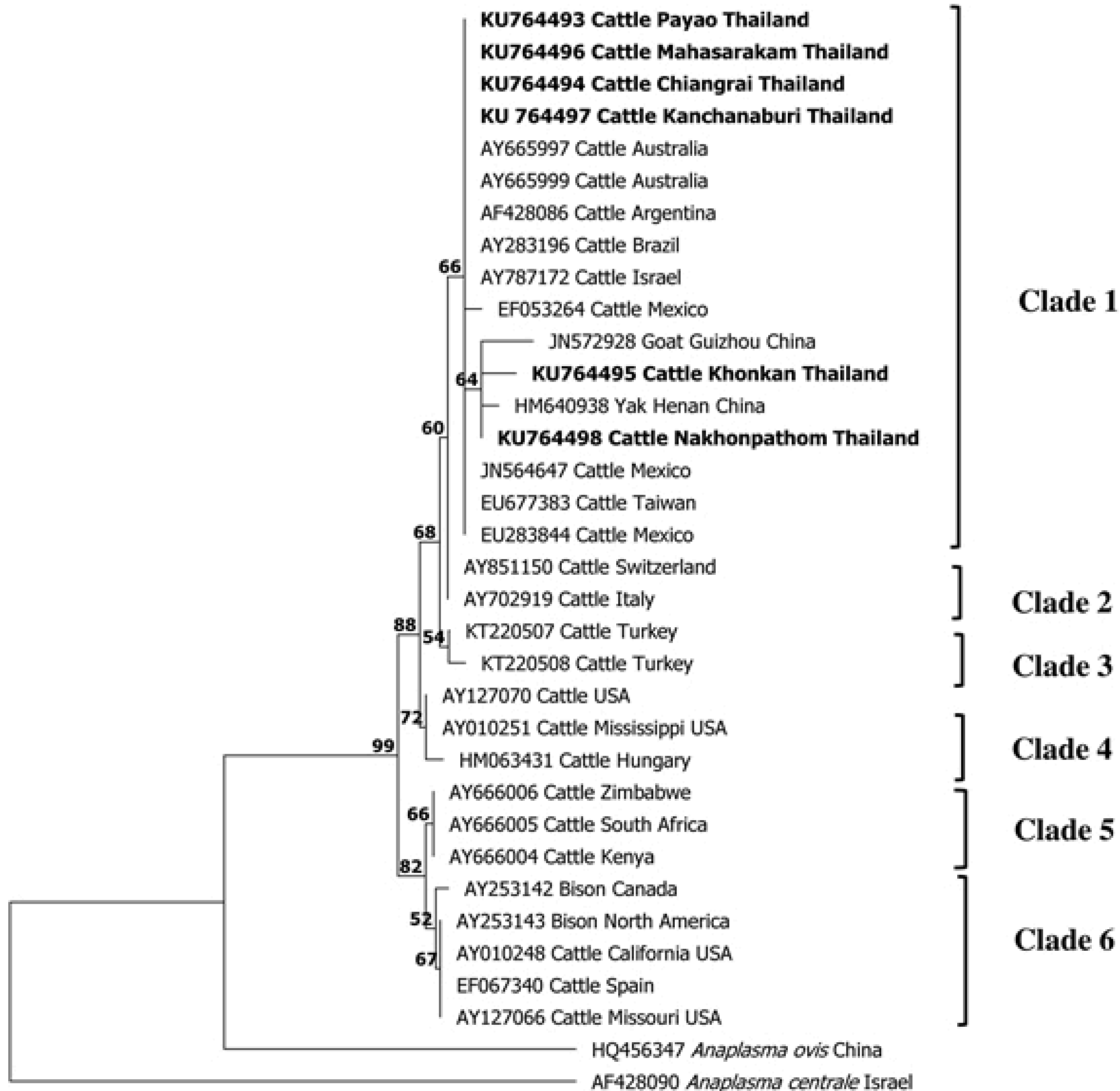
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AY665999 Cattle Australia
AF428086 Cattle Argentina
AY283196 Cattle Brazil
AY787172 Cattle Israel
EF053264 Cattle Mexico
JN572928 Goat Guizhou China
KU764495 Cattle Khonkan Thailand
HM640938 Yak Henan China
KU764498 Cattle Nakhonpathom Thailand
JN564647 Cattle Mexico
EU677383 Cattle Taiwan
EU283844 Cattle Mexico
AY851150 Cattle Switzerland
AY702919 Cattle Italy
KT220507 Cattle Turkey
KT220508 Cattle Turkey
AY127070 Cattle USA
AY010251 Cattle Mississippi USA
HM063431 Cattle Hungary
AY666006 Cattle Zimbabwe
AY666005 Cattle South Africa
AY666004 Cattle Kenya
AY253142 Bison Canada
AY253143 Bison North America
AY010248 Cattle California USA
EF067340 Cattle Spain
AY127066 Cattle Missouri USA
HQ456347 Anaplasma ovis China
AF428090 Anaplasma centrale Israel
Clade 1
Clade 2
Clade 3
Clade 4
Clade 5
Clade 6
66
64
60
68
88
54
72
99
66
82
52
67
0.02

Table 1. Summary of *Babesia* spp., *Theileria orientails* and *Anaplasma marginale* single species infections in cattle detected using PCR assay.

Region	Province	No. of tested cattle	*T. orientalis*		*B. bigemina*		*B. bovis*		*A. marginale*	
			Positive	% (CI)	Positive	% (CI)	Positive	% (CI)	Positive	% (CI)
North	Chiangrai	55	1	1.8 (0.3-9.6)	0	0	0	0	11	20.0 (11.5-32.3)
	Payao	14	1	7.1 (1.3-31.5)	0	0	0	0	0	0
	Mae hong son	60	1	1.7 (0.3-8.9)	5	8.3 (3.6-18.1)	2	3.3 (0.9-11.4)	2	3.3 (0.9-11.4)
Northeast	Khon kan	50	1	2.0 (0.4-10.5)	4	8.0 (3.2-18.8)	0	0	6	12.0 (5.6-23.8)
	Mahasarakam	85	31	36.5 (27.0-47.1)	12	14.1 (8.3-23.1)	1	1.2 (0.2-6.4)	2	2.4 (8.6-8.2)
	Loei	65	17	26.2 (17.0-37.9)	4	6.2 (2.4-14.8)	0	0	2	3.1 (0.8-10.5)
Central and western	Kanchanaburi	125	4	3.2 (1.3-7.9)	2	1.6 (0.4-5.6)	1	0.8 (0.1-4.4)	33	26.4 (19.5-34.8)
	Rachaburi	90	4	4.4 (1.7-10.9)	0	0	2	2.2 (0.6-7.7)	14	15.6 (9.5-24.4)
	Nakhonpathom	64	0	0	6	9.4 (4.4-19.0)	1	1.6 (0.3-8.3)	18	28.1 (18.6-40.1)
	Total	608	60	9.9 (7.7-12.5)	33	5.4 (3.9-7.5)	7	1.2 (0.5-2.3)	88	14.5 (11.9-17.5)

Table 2. Multiple infections in beef cattle out of 608 animals sampled.

Parasite infection	Frequency	Infection rate (%)
Two pathogens		
A. marginale and T. orientalis	59	9.7
A. marginale and *B. bigemina*	29	4.8
B. bigemina and *T. orientalis*	28	4.6
A. marginale and *B. bovis*	24	3.9
B. bigemina and *B. bovis*	9	1.5
B. bovis and *T. orientalis*	1	0.2
Three pathogens		
A. marginale, B. bigemina and *T. orientalis*	32	5.3
A. marginale, B. bigemina and *B. bovis*	28	4.6
A. marginale, B. bovis and *T. orientalis*	8	1.3
B. bigemina, B. bovis and *T. orientalis*	3	0.5
Four pathogens		
A. marginale, B. bovis, B. bigemina and *T. orientalis*	8	1.3
Total	229	37.7