Genetic analysis of *Babesia* isolates from cattle with clinical babesiosis in Sri Lanka

Running title: Clinical babesiosis in cattle in Sri Lanka

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Abstract

Bovine babesiosis is a serious threat to the cattle industry. We prepared blood DNA samples from 13 cattle with clinical babesiosis from the Badulla (n=8), Jaffna (n=3), and Kilinochchi (n=2) districts in Sri Lanka. These DNA samples tested positive in PCR assays specific for *Babesia bovis* (n=9), *B. bigemina* (n=9), and *B. ovata* (n=1). Twelve cattle were positive for *B. bovis* and/or *B. bigemina*. One cow was negative for the tested *Babesia* species, but positive for *Babesia* on microscopic examination; the phylogenetic positions of *18S rRNA* and cytochrome oxidase subunit III gene sequences suggested that the cow was infected with *Babesia* sp. Mymensingh, which was recently reported in a healthy cow in Bangladesh. We then developed a novel *Babesia* sp. Mymensingh-specific PCR assay, and obtained positive results for one other sample. Analysis of gene sequences from the cow with positive *B. ovata*-specific PCR results demonstrated that the animal was not infected with *B. ovata*, but with *Babesia* sp. Hue-1, which was recently reported in asymptomatic cattle in Vietnam. The virulence of *Babesia* sp. Hue-1 is unclear, as the cow was co-infected with *B. bovis* and *B. bigemina*. However, *Babesia* sp. Mymensingh probably causes severe clinical babesiosis, as it was the sole *Babesia* species detected in a clinical case. The present study revealed the presence of two bovine *Babesia* species not previously reported in Sri Lanka, plus the first case of severe bovine babesiosis caused by a *Babesia* species other than *B. bovis*, *B. bigemina*,

and *B*. *divergens*.

Keywords: *Babesia*, cattle, clinical babesiosis, Sri Lanka

INTRODUCTION

Clinical bovine babesiosis is mainly caused by *Babesia bovis* and *B. bigemina* in the tropics and subtropics (1), while *B. divergens* causes clinical bovine babesiosis in Europe (2). *Babesia* sporozoites injected by infected tick vectors directly invade the host's red blood cells (RBCs) and develop into merozoites (3, 4). Merozoites egress from the infected RBCs, causing massive intravascular hemolysis that leads to clinical signs such as fever, anemia, hemoglobinuria, and jaundice (1). Additionally, bovine babesiosis caused by *B. bovis* is characterized by neurological and respiratory syndromes because of the cytoadherence of infected RBCs in capillary beds of vital organs such as the brain and lungs (1, 5). Early treatment with anti-babesial agents is essential for recovery, and no or delayed treatment may result in severe babesiosis that causes death (6, 7). Thus, bovine babesiosis results in huge economic losses to the cattle industry because of treatment and tick control costs, production losses, and animal mortality (8). Moreover, babesiosis disrupts international cattle trade, as the OIE regulations stipulate rules for exporting cattle from countries in which bovine babesiosis is endemic (9).

Disease development in infected cattle is affected by multiple factors, including age, management practices, immunity, and breed (1). Young animals less than 9 months old are usually resistant to clinical babesiosis, while adults are not (10, 11). The immunity acquired

71 by young animals protects them from developing clinical babesiosis when they become
72 infected as adults. In addition, *Bos indicus* are relatively resistant to clinical babesiosis
73 compared with *Bos taurus* (12).

74 Cattle in several endemic countries are immunized against *B*. *bovis* and *B*. *bigemina*
75 via live-attenuated vaccines (13). The attenuation of *B*. *bovis* and *B*. *bigemina* is performed by
76 serially passaging the organisms in splenectomized calves (1). However, the global use of live
77 vaccines is limited due to the expense and time required to produce the vaccines, vaccine
78 breakthrough due to strain variations, and risk of contamination with other bovine blood
79 pathogens (14, 15).

80 In addition to *B*. *bovis*, *B*. *bigemina*, and *B*. *divergens*, several other species of
81 *Babesia* are known to infect cattle, such as *B*. *ovata* (16), *B*. *occultans* (17), *B*. *major* (18), and
82 several unclassified species, including *Babesia* sp. Oshima (19), *Babesia* sp. Kashi (20),
83 *Babesia* sp. Hue-1 (21), *Babesia* sp. Mymensingh (22), and *Babesia* species isolated in South
84 Africa (23, 24). Most of these *Babesia* species have low pathogenicity; however, the virulence
85 of the remaining species is unknown, as they have only been reported in apparently healthy
86 animals (21, 22). To determine the virulence of the *Babesia* species with unknown
87 pathogenicity, there is a need for experimental infections or investigations to identify the
88 *Babesia* species present in cattle with clinical babesiosis.

89 Clinical babesiosis is common among cattle in the tropical country of Sri Lanka. A

few decades ago, clinical cases of bovine babesiosis were concentrated mainly in the wet zone of Sri Lanka, whereas the disease was uncommon in the dry zone (25). This discrepancy was due to the variation in cattle breeds and management practices between the wet and dry zones (12, 26); most cattle in the wet zone were temperate breeds (*Bos taurus*) that were managed intensively, while the dry zone contained mostly extensively managed local cattle (*Bos indicus*) (27). However, farmers in the dry zone have recently started to maintain temperate breeds and their crosses and adopt an intensive management system, leading to an increased incidence of clinical babesiosis. Hence, clinical babesiosis in cattle is now common in both the wet and dry zones (28). Although live vaccines against both *B. bovis* and *B. bigemina* are available in Sri Lanka, only a few hundred claves are vaccinated annually (29, 30).

A recent series of molecular epidemiological surveys were conducted in Sri Lanka to detect and genetically characterize hemoprotozoan parasites in apparently healthy cattle (31–35). In contrast, genetic techniques have never been used to investigate clinical cases of bovine babesiosis in Sri Lanka. Therefore, in the present study, we employed molecular tools to identify the *Babesia* species present in cattle with clinical babesiosis in Sri Lanka.

MATERIALS AND METHODS

Animals and blood samples. Between June and December 2017, approximately 2 ml samples of whole blood were obtained from the jugular veins of 13 cattle with high fever and hemoglobinuria in the Badulla (n=8), Jaffna (n=3), and Kilinochchi (n=2) districts of Sri Lanka (Fig. S1) using Vacutainer tubes containing EDTA (NIPRO, Osaka, Japan) (Table 1). The Jaffna and Kilinochchi districts are located in the dry zone, while the sampling locations within the Badulla district were located in the wet zone. The affected animals were either Friesians, Jerseys, or Jersey and Sahiwal crosses, and their ages ranged from 3 to 6 years. None of the affected animals were vaccinated against *B*. *bovis* and/or *B*. *bigemina*. Immediately after sampling, the animals were treated with diminazene aceturate and long-acting oxytetracycline. Blood samples were analyzed in a commercial laboratory to determine the hemoglobin concentration (HGB), hematocrit (HCT), and RBC count. DNA samples were extracted from blood samples using a commercial kit (QIAamp DNA Blood Mini Kit; Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions, and stored at −30ºC until use. All animal procedures were approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (approval number: 29-53).

126 **Diagnostic PCR assays.** A previously described PCR assay based on the
127 rhoptry-associated protein 1 gene (36) was used to detect *B*. *bovis*, while apical membrane
128 antigen 1 (*ama-1*) gene-based PCR assays were employed to detect *B*. *bigemina* (37) and *B*.
129 *ovata* (38). All parasite species were detected by single-step PCR, although *B*. *bovis*- and *B*.
130 *bigemina*-specific PCR assays were originally described as nested PCR assays. The list of
131 primer sequences used in the present study is provided in Table S1.
132
133 **Cloning and sequencing.** An amplicon from the *ama-1* PCR assay targeting *B*.
134 *ovata* was cloned and sequenced as previously described (21), as this *Babesia* species has not
135 been reported in Sri Lanka. In addition, the *18S rRNA* and cytochrome oxidase subunit III
136 (*cox3*) gene sequences of babesial origin from a DNA sample that was PCR-positive for *B*.
137 *ovata* and from a sample that was PCR-negative for all three *Babesia* species tested in the
138 present study were amplified by PCR using sets of common primers. Briefly, a 25-µl reaction
139 mix containing 1 µl DNA, 1× PCR buffer (Toyobo, Osaka, Japan), 400 µM of each dNTP
140 (Toyobo), 0.4 µM of each forward primer (*18S rRNA*,
141 5′-CATTACAACAGTTATAGTTTCTTTGG-3′ (21); *cox3*,
142 5′-TCAACAAAATGCCAATATGTTCCAA-3′) and reverse primer (*18S rRNA*,
143 5′-CTAGGCATTCCTCGTTCATGATTTAG-3′; *cox3*,
144 5′-TACAAAGTGCATCTTTGGGAGAAG-3′), 0.5 µl of 1 U/µl KOD FX Neo DNA

polymerase (Toyobo), and 4 μl of distilled water was subjected to an initial pre-denaturation step at 94ºC for 2 min and then to 35 cycles of denaturation at 94ºC for 10 s, annealing at 58ºC (*18S rRNA*) or 64ºC (*cox3*) for 30 s, and extension at 68ºC for 90 s. The PCR amplicons were cloned into a PCR 2.1 plasmid vector (TOPO, Invitrogen, Carlsbad, CA), and then sequenced as previously described (37).

Development of a PCR assay specific to *Babesia* sp. Mymensingh. One animal that produced negative results in the PCR assays for *B. bovis*, *B. bigemina*, and *B. ovata* was determined to be infected with *Babesia* sp. Mymensingh (22) based on an *18S rRNA* sequence. A pair of common forward (5′-TGGACCAGGTACATGATCAAGT-3′) and reverse (5′-AATCATCGTGCTGACGACCCTTC-3′) PCR primers (37) was used to amplify the 1372-bp *ama-1* gene fragment from *Babesia* sp. Mymensingh, as described for *18S rRNA* or *cox3* amplification, except that the annealing temperature was changed to 62ºC. The amplicon was cloned and sequenced. The newly generated *ama-1* gene sequence and those already available in the NCBI GenBank database were subjected to multiple alignment, and a set of forward (5′-TGGCGCCGACTTCCTGGAGCCCATCTCCAA-3′) and reverse (5′-AGCTGGGGCCCTCCTTCGATGAACCGTCGG-3′) primers specific to *Babesia* sp. Mymensingh was designed. A 10-μl PCR mixture containing 1 μl DNA, 1× PCR buffer (Applied Biosystems, Branchburg, NJ), 200 μM of each dNTP (Applied Biosystems), 0.5 μM

of each forward and reverse primer, 0.1 µl of 5 U/µl AmpliTaq Gold DNA polymerase (Applied Biosystems), and 5.9 µl of distilled water was subjected to initial enzyme activation at 95ºC for 5 min, followed by 40 cycles of denaturing at 95ºC for 30 s, annealing at 64ºC for 30 s, and extension at 72ºC for 30 s. After a final elongation step at 72ºC for 7 min, the PCR products were resolved by gel electrophoresis, and then visualized under UV illumination. Detection of an approximately 371-bp band was considered positive. The specificity of the PCR assay was evaluated using a panel of DNA samples derived from several bovine blood pathogens and uninfected cattle (38, 39). The *Babesia* sp. Mymensingh-specific PCR assay was then used to screen all 13 cattle DNA samples analyzed in the present study.

Sequencing and phylogenetic analyses. The identity scores among gene sequences were determined by the EMBOSS NEEDLE online program (http://www.bioinformatics.nl/cgi-bin/emboss/needle). The *18S rRNA*, *cox3*, and *ama-1* gene sequences determined in the present study and those retrieved from GenBank were aligned using the MAFFT software program (40). MEGA software (41) was then used to construct three separate maximum likelihood phylogenetic trees based on Tamura-Nei (*18S rRNA* and *cox3*) (42) or General Time Reversible (*ama-1*) (43) substitution models.

Accession numbers. The gene sequences determined in the present study were

183 registered with GenBank under the accession numbers LC385886–LC385894.

184

185 **RESULTS**

186

187 A total of 13 cattle with high fever and hemoglobinuria were sampled in three
188 districts: Badulla, Kilinochchi, and Jaffna. Clinical examination determined that five of the
189 eight animals sampled in the Badulla district were anemic, whereas all five of the animals
190 sampled in the Kilinochchi and Jaffna districts were anemic (Table 1). Five of the affected
191 animals also showed nervous system abnormalities such as incoordination. The anemic
192 animals each had a HGB concentration, HCT, and RBC count below the lower limits of the
193 reference ranges for these parameters in healthy cattle (8 g/dl, 24%, and 5×10^6/μl,
194 respectively) (44). Two animals in the Kilinochchi district (C6 and C7) and one animal in the
195 Badulla district (S) with very low HGB concentrations, HCT, and RBC counts died despite
196 treatment with diminazene aceturate and oxytetracycline, while the remaining animals
197 recovered following treatment. PCR assays revealed that 12 of the 13 DNA samples were
198 positive for *B. bovis* and/or *B. bigemina* (Table 2). *B. bovis* and *B. bigemina* were each
199 detected in nine animals, while six animals were positive for both *Babesia* species. The result
200 of the *B. ovata*-specific *ama-1* PCR assay was positive in only one animal (C8 in the Jaffna
201 district).

202 Cow R from the Badulla district was PCR-negative for all three *Babesia* species
203 tested, but microscopic examination revealed *Babesia* piroplasms in a thin blood smear

prepared from this animal (Fig. 1). The length and width of the paired pyriforms were 2.25–3.04 μm and 1.58–2.20 μm, respectively, whereas the ring forms were 1.52–1.97 μm in diameter. The paired pyriforms formed an obtuse angle that reached 180º in some cases. Single forms were often elongated or irregularly shaped. *18S rRNA* is the most commonly used molecular marker for species identification of eukaryotes, including *Babesia* (18, 20, 45–47). Therefore, in an attempt to identify the species of *Babesia* detected in cow R, a 1385-bp *18S rRNA* sequence was isolated from cow R (GenBank accession number LC385886) and compared with the *Babesia* sequences in the GenBank database. The analyzed gene sequence shared 99.6% identity values with the *Babesia* sp. Mymensingh sequence (GenBank accession number MF576177) that was recently reported from an asymptomatic cow in Bangladesh (22), followed by 97.8% identity with a *B. bigemina* sequence (GenBank accession number FJ426361) (48); this confirmed that the *Babesia* species detected in cow R was *Babesia* sp. Mymensingh. In the *18S rRNA* phylogeny based on a 1310-bp alignment with no gaps, the *Babesia* sp. Mymensingh sequence determined in the present study clustered together with that previously reported in Bangladesh (22) and formed a sister clade to *B. bigemina* (Fig. 2).

As *B. ovata* has not been previously reported in Sri Lanka, the amplicon of the PCR assay targeting *B. ovata* from cow C8 was cloned and sequenced. The resultant *ama-1* gene fragment (GenBank accession number LC385892) shared only 93.3% identity with the *B.*

223 *ovata* sequence (GenBank accession number AB634843) (37), while the sequence shared
224 97.4%–98.4% identity scores with *ama-1* sequences (GenBank accession numbers
225 LC125412–LC125415) from recently reported *Babesia* sp. Hue-1 in Vietnam (21). The *18S*
226 *rRNA* amplified from cow C8 included *B. bigemina* sequences (data not shown), as well as a
227 1381-bp sequence (GenBank accession number LC385887) that shared a high identity score
228 (99.0%) with a *Babesia* sp. Hue 1 sequence (GenBank accession number LC125456) (21).
229 These findings suggest that cow C8 was infected with *Babesia* sp. Hue-1, as the identity
230 scores shared between the *18S rRNA* and *ama-1* gene sequences from cow C8 and *Babesia* sp.
231 Hue-1 are comparable to the identity scores shared among these sequences from different
232 isolates of other bovine *Babesia* species (49–52). Phylogenetically, *Babesia* sp. Hue-1 from
233 cow C8 formed a sister clade to the common ancestor of the *B. bigemina* clade and the
234 *Babesia* sp. Mymensingh clade (Fig. 2). Moreover, in a phylogeny based on a short alignment
235 (635 bp) of *18S rRNA*, *Babesia* sp. Hue-1 sequences from cow C8 in Sri Lanka and that
236 reported in Vietnam occurred together and formed a sister clade to the clade formed by
237 sequences from *Babesia* species reported in China (GenBank accession number AY603403)
238 (18) and Korea (GenBank accession number AY081192) (53) (Fig. S2).

239 In addition to *18S rRNA*, phylogenies based on mitochondrial genes are widely used
240 for the identification of species, including *Babesia* species (54–57). Therefore, to further
241 investigate the phylogenetic positions of *Babesia* sp. Mymensingh and *Babesia* sp. Hue-1,

242 *cox3* gene sequences (556 bp) were amplified from DNA samples that were positive for these
243 *Babesia* species (from cows R and C8, respectively). The *cox3* sequence from *Babesia* sp.
244 Mymensingh (GenBank accession number LC385889) shared 93.5% identity with *B*.
245 *bigemina* (GenBank accession number LK054939) (58) and *B*. *ovata* (GenBank accession
246 number LC146482) (52) sequences, and formed a phylogenetic sister clade to *B*. *bigemina*
247 (Fig. 3). Only the *cox3* sequence from *Babesia* sp. Hue-1 (GenBank accession number
248 LC385890) was isolated from cow C8. The *cox3* sequence from *Babesia* sp. Hue-1 shared
249 94.9%, 94.1%, and 93.5% identity with sequences from *Babesia* sp. (Wenchuan, China)
250 (GenBank accession number JN859545) (56), *B*. *ovata* (GenBank accession number
251 LC146482), and *B*. *bigemina* (GenBank accession number LK054939), respectively, and
252 formed a sister clade to the clade formed by *Babesia* species reported in China (Wenchuan)
253 and Korea (Fig. 3).

254 The *ama-1* gene is an attractive target for the development of species-specific PCR
255 assays for detecting *Babesia* parasites, as the gene is conserved within a given *Babesia*
256 species, but is diverse between species (37, 59–61). Therefore, a 1372-bp *ama-1* gene
257 fragment (GenBank accession number LC385893) was isolated from *Babesia* sp.
258 Mymensingh to develop a specific PCR assay to detect this *Babesia* species in the DNA
259 samples. The gene sequence shared 83.9% and 83.3% identity with *B*. *bigemina* (GenBank
260 accession number AB481200) (62) and *B*. *ovata* (GenBank accession number AB634843)

261 (37) sequences, respectively. The PCR assay developed based on the *ama-1* gene amplified
262 only *Babesia* sp. Mymensingh, while no amplicons were observed in DNA samples from
263 several other bovine blood pathogens and cattle DNA, confirming its specificity (Fig. 4A).
264 When this PCR assay was employed to screen all 13 cattle DNA samples, *Babesia* sp.
265 Mymensingh was detected not only in cow R, but also in cow V (Fig. 4B). PCR amplicons
266 from cows R and V were cloned and sequenced. The 371-bp *ama-1* sequences from cows R
267 and V (GenBank accession number LC385894) were identical to each other and to the long
268 *ama-1* gene fragment initially isolated from cow R (GenBank accession number LC385893).
269 Phylogenetic trees based on the *ama-1* gene are used to investigate the evolutionary
270 relationships of apicomplexan parasites, such as species of *Plasmodium* and *Babesia* (61,
271 63–65). In a phylogenetic construction used to investigate the positions of *Babesia* sp.
272 Mymensingh and *Babesia* sp. Hue-1, the *ama-1* sequences of *Babesia* sp. Mymensingh
273 occurred distant to the sequences of *B. bigemina* (Fig. 5). In addition, the *Babesia* sp. Hue-1
274 *ama-1* sequence generated in the present study clustered with those previously reported in
275 Vietnam and formed a sister clade to *B. ovata*. To further confirm that cow V was infected
276 with *Babesia* sp. Mymensingh, *18S rRNA* and *cox3* sequences were amplified, cloned, and
277 sequenced. The *18S rRNA* sequence (GenBank accession number LC385888) from cow V
278 shared 99.6% identity with the *Babesia* sp. Mymensingh sequence from cow R (GenBank
279 accession number LC385886), whereas the *cox3* sequence from cow V (GenBank accession

number LC385891) was identical to that from cow R (GenBank accession number LC385889), confirming that cow V was also infected with *Babesia* sp. Mymensingh.

DISCUSSION

Various species of *Babesia* infect a wide range of host species worldwide, including wild and domestic animals, humans, birds, and reptiles (66). The *Babesia* species that infect cattle are of great economic importance, as they cause severe clinical diseases leading to significant production losses (1). Among them, *B. bovis*, *B. bigemina*, and *B. divergens* are highly virulent species that cause a severe form of bovine babesiosis. Although bovine babesiosis is very common in Sri Lanka, a detailed examination of clinical cases using molecular techniques has never been carried out in this country. Therefore, in the present study, we used molecular diagnostic tools to investigate clinical babesiosis in Sri Lanka.

Anemia was detected in 10 of the 13 animals with fever and hemoglobinuria investigated in the present study, while three animals from the Badulla district were not anemic at the time of sampling, suggesting that these animals were sampled in the early stage of disease development. Three animals with very low RBC indices died even after treatment with an anti-babesial drug (diminazene aceturate), suggesting that early veterinary intervention is of paramount importance in bovine babesiosis (7). The neurological signs observed in five animals might have been due to *B. bovis* infection, which can cause cerebral babesiosis in cattle (1, 5). However, such neurological signs can also occur in anemic animals due to hypoxia-related brain injury caused by low hemoglobin levels (67, 68). Post-mortem

examination could have clarified whether the actual cause of the neurological signs in cows S and C7 was cerebral babesiosis or hypoxic brain injury. Unfortunately, however, post-mortem examinations were not carried out for any of the animals that died.

The PCR assays and sequencing analyses detected *B*. *bovis* and *B*. *bigemina*, as well as two other *Babesia* species (*Babesia* sp. Mymensingh and *Babesia* sp. Hue-1) that had not been previously reported in Sri Lanka. In addition to *Babesia* parasite species, previously described species-specific PCR assays (69–72) determined that the samples contained *Theileria annulata*, *Theileria. orientalis*, *Trypanosoma theileri*, and *Anaplasma marginale* (Tables S1 and S2). However, the involvement of these parasite species in the animals' clinical disease was unclear, as none of these parasite species induce hemoglobinuria.

Based on morphological observation, *Babesia* sp. Mymensingh can be classified as a large *Babesia*. However, the piroplasms were morphologically different from those of *B*. *bigemina*, as the paired pyriforms of the latter usually form an acute angle (73). In addition, the size of the ring forms in *Babesia* sp. Mymensingh was smaller than that in *B*. *bigemina*. *Babesia* sp. Mymensingh was initially identified based on an *18S rRNA* sequence from an apparently asymptomatic cow in Bangladesh (22). In contrast, the parasite in the present study was detected as the sole *Babesia* species in a cow (R) with typical signs of clinical babesiosis, suggesting that *Babesia* sp. Mymensingh is a virulent species.

The phylogenetic position of *Babesia* sp. Mymensingh was further analyzed in a

cox3-based phylogeny, in which it formed a sister clade to *B*. *bigemina*. The sister clades formed in *18S rRNA* and *cox3* phylogenies may identify *Babesia* sp. Mymensingh as a new genotype of *B*. *bigemina*. However, the low identity scores shared between the *18S rRNA*, *cox3*, and *ama-1* gene sequences from these parasite species, the *ama-1* phylogeny in which *Babesia* sp. Mymensingh formed a separate clade, and the morphological differences confirm that *Babesia* sp. Mymensingh is a distinct *Babesia* species. The PCR detection of *Babesia* sp. Mymensingh in an additional cow suggested that *Babesia* sp. Mymensingh infection might be common among cattle in Sri Lanka. *Babesia* sp. Mymensingh may have a wide distribution, as this parasite species was also detected in Bangladesh. Therefore, the PCR assay developed in the present study will be a useful diagnostic tool for specific detection of *Babesia* sp. Mymensingh in different geographical territories.

Cow R that was infected with *Babesia* sp. Mymensingh was reexamined 1 week after sampling. No hemoglobinuria was present, and the anemia was milder than previously. Furthermore, laboratory examination showed improvements in the HGB concentration (8 g/dl), HCT (23.4%), and RBC count (4.53×10^6/μl) compared with the first sampling, indicating that diminazene aceturate is effective against *Babesia* sp. Mymensingh. However, *Babesia* sp. Mymensingh might have implications for the immune control and diagnostics of bovine babesiosis. For example, live-attenuated vaccines are used in some endemic countries, including Sri Lanka, to immunize cattle against bovine babesiosis caused by *B*. *bovis* and *B*.

340 *bigemina* (13). If *Babesia* sp. Mymensingh is not immunologically cross-reactive to these
341 parasite species, especially to *B. bigemina*, *Babesia* sp. Mymensingh may cause clinical
342 disease in vaccinated animals. However, if they are immunologically cross-reactive parasites,
343 the sero-diagnostic techniques commonly used in epidemiological surveys might generate
344 unreliable data.

345 The PCR assay targeting *B. ovata* detected *Babesia* sp. Hue-1. Phylogenetically, *18S*
346 *rRNA* from *Babesia* sp. Hue-1 formed a clade that was clearly separate from the other *Babesia*
347 species, identifying *Babesia* sp. Hue-1 as a separate *Babesia* species. The morphology of
348 *Babesia* sp. Hue-1 was not analyzed, as the blood smear from the infected cattle was unfit for
349 microscopic examination. Even if high-quality blood smears had been available, microscopic
350 detection might have been impossible, as the animal was co-infected with *B. bovis* and *B.*
351 *bigemina*. *Babesia* sp. Hue-1 was recently reported in Vietnam based on *18S rRNA* and *ama-1*
352 sequences from healthy cattle (21). Therefore, the clinical significance of *Babesia* sp. Hue-1
353 remains unknown.

354 In conclusion, the present study demonstrated the presence of two *Babesia* species
355 not previously detected in Sri Lanka, and also identified severe clinical bovine babesiosis
356 caused by a *Babesia* species other than *B. bovis*, *B. bigemina*, and *B. divergens*. Priorities in
357 *Babesia* research include isolation of the newly detected *Babesia* species, experiments to
358 investigate their virulence in different cattle breeds and immunological cross reactivity with *B.*

359 *bovis* and *B*. *bigemina*, and identification of specific tick vectors.

360

361 **Appendix A**

362 **Supplemental file 1**

363

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591

592

593 **FIGURE LEGENDS**

594

595 **Fig. 1.** Morphology of the *Babesia* parasites in cow R. A Giemsa-stained thin blood smear
596 prepared from cow R was observed under a light microscope. (A) Paired pyriforms, (B) ring
597 forms, and (C) elongated and irregularly shaped single forms of *Babesia* parasites were
598 detected in cow R.

599

600 **Fig. 2.** Phylogeny of *18S rRNA*. A maximum-likelihood phylogeny was constructed using *18S*
601 *rRNA* sequences determined in the present study and those retrieved from GenBank. The gene
602 sequences determined in the present study are indicated by the boldface type letters. Note that
603 the *Babesia* sp. Mymensingh sequence determined in the present study and that previously
604 reported from Bangladesh clustered together and formed a sister clade to *B*. *bigemina*.
605 Additionally, the *Babesia* sp. Hue-1 occurred separately, forming a sister clade to the
606 common ancestor of *Babesia* sp. Mymensingh and *B*. *bigemina*.

607

608 **Fig. 3.** Phylogeny of *cox3*. A maximum-likelihood phylogeny was constructed using *cox3*
609 sequences from *Babesia* sp. Mymensingh and *Babesia* sp. Hue-1 and those available in the
610 GenBank database. The gene sequences determined in the present study are indicated by the
611 boldface type letters. Note that *Babesia* sp. Mymensingh formed a sister clade to *B*. *bigemina*,

612 whereas *Babesia* sp. Hue-1 formed a sister clade to *Babesia* sp. (Wenchuan, China)/*Babesia*
613 sp. (Lushi, China).
614
615 **Fig. 4.** Development of a *Babesia* sp. Mymensingh-specific PCR assay. A PCR assay specific
616 to *Babesia* sp. Mymensingh was developed. (A) Specificity testing. The specificity of the
617 newly developed PCR assay was tested using DNA samples from *Babesia* sp. Mymensingh, *B*.
618 *bigemina*, *B*. *bovis*, *B*. *ovata*, *B*. *divergens*, *Babesia* sp. Hue-1, *Theileria annulata*, *Th. parva*,
619 *Th. orientalis*, *Trypanosoma evansi*, *Tr. theileri*, *Tr. brucei*, *Anaplasma marginale*, *A*. *bovis*,
620 and uninfected cattle (lanes 1–15, respectively). M indicates the 100-bp DNA marker. Note
621 that the amplicon with the expected size was observed only with *Babesia* sp. Mymensingh.
622 (B) Screening of 13 clinical samples for *Babesia* sp. Mymensingh. The PCR assay specific to
623 *Babesia* sp. Mymensingh was used to screen DNA samples from 13 clinical cases. M and NC
624 indicate the 100-bp DNA marker and non-template control, respectively. Note that *Babesia* sp.
625 Mymensingh was also detected in cow V.
626
627 **Fig. 5.** Phylogeny of *ama-1*. The *ama-1* sequences from *Babesia* sp. Mymensingh from cows
628 R and V (amplified by *Babesia* sp. Mymensingh-specific PCR) and from *Babesia* sp. Hue-1
629 from cow C8 (amplified by *B*. *ovata* PCR) together with those retrieved from GenBank were
630 used to construct a maximum-likelihood phylogeny. The gene sequences determined in the

present study are indicated by the boldface type letters. Note that the *Babesia* sp. Hue-1 sequences clustered with those previously reported in Vietnam and formed a sister clade to *B. ovata,* while the *Babesia* sp. Mymensingh formed a separate clade.

Table 1. Clinical presentation of bovine babesiosis in Sri Lanka

No.	District	Animal ID	Breed	Sex	Age (years)	Clinical signs				RBC indices[a]		
						Temperature (ºC)	Anemia	Hemoglobinuria	Nervous signs	HGB	HCT	RBC
1	Badulla	I	Jersey	F	4	40.6	Y	Y	Y	NT	NT	NT
2	Badulla	L1	Friesian	F	3	41.4	N	Y	N	8.4	22.6	5.65
3	Badulla	L2	Jersey	F	4	41.1	N	Y	N	9.6	27.6	5.94
4	Badulla	R	Friesian	F	4	41.1	Y	Y	N	6.8	19.6	3.94
5	Badulla	S[b]	Jersey	F	5	39.4	Y	Y	Y	2.8	7.9	1.38
6	Badulla	T1	Jersey	F	4	41.1	Y	Y	Y	8.7	24	5.59
7	Badulla	T2	Jersey	F	4	41.1	N	Y	N	9.4	25.7	5.51
8	Badulla	V	Jersey	F	5	42.2	Y	Y	N	NT	NT	NT
9	Jaffna	C2	Jersey	F	4	40.6	Y	Y	N	6.8	21.9	3.88
10	Jaffna	C5	Jersey	F	5	40	Y	Y	Y	6.7	21.5	3.5
11	Kilinochchi	C6[b]	Jersey × Sahiwal	F	6	39.4	Y	Y	N	2.83	10.6	2.22
12	Kilinochchi	C7[b]	Jersey	F	3	41.1	Y	Y	Y	4.8	13.3	3.03
13	Jaffna	C8	Jersey × Sahiwal	F	6	40	Y	Y	N	NT	NT	NT

ID, identification; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; F, female; Y, yes (observed); N, no (not observed); NT, not tested.

[a] RBC indices, including the HGB concentration (g/dl), HCT (%), and RBC count ($\times 10^6$/µl), were measured for 10 of the 13 animals.

[b] These animals (S, C6, and C7) died despite treatment with diminazene aceturate.

Table 2. PCR detection of *B. bovis, B. bigemina, and B. ovata* in clinical cases of bovine babesiosis in Sri Lanka

No.	Animal ID	*Babesia bovis*	*Babesia bigemina*	*Babesia ovata*
1	I	+	–	–
2	L1	–	+	–
3	L2	–	+	–
4	R[a]	–	–	–
5	S	+	+	–
6	T1	+	+	–
7	T2	+	+	–
8	V	+	+	–
9	C2	–	+	–
10	C5	+	–	–
11	C6	+	–	–
12	C7	+	+	–
13	C8	+	+	+
Total		9	9	1

PCR, polymerase chain reaction; ID, identification; +, positive; –, negative

[a]Animal R was negative for all three *Babesia* species tested in the present study.

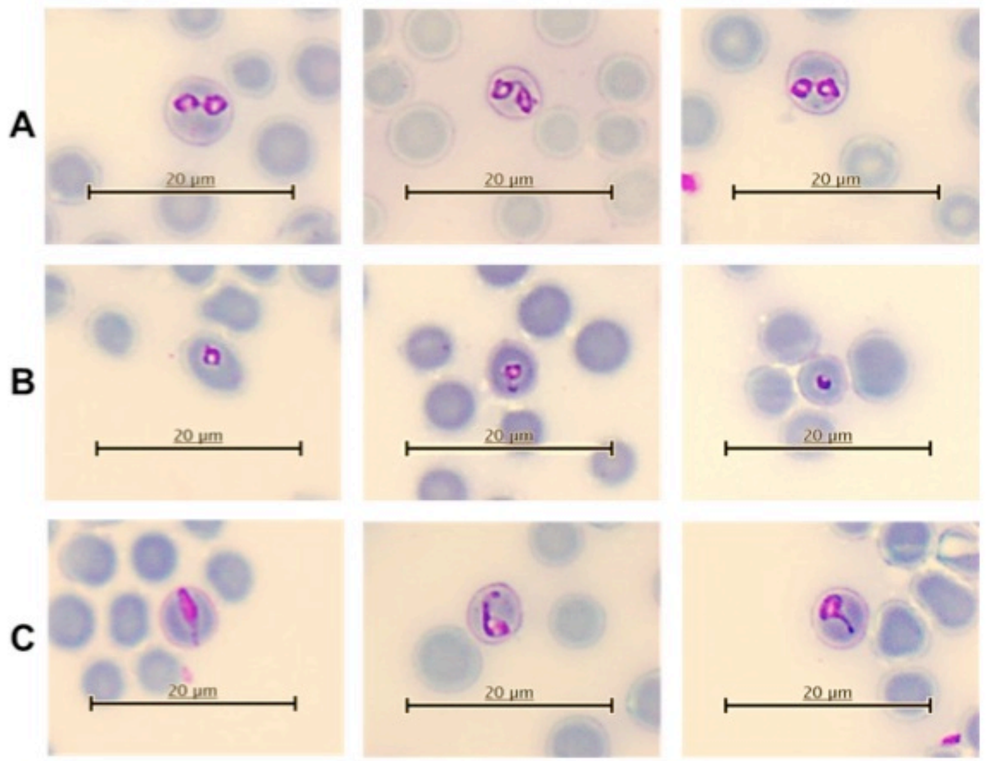

Fig. 1

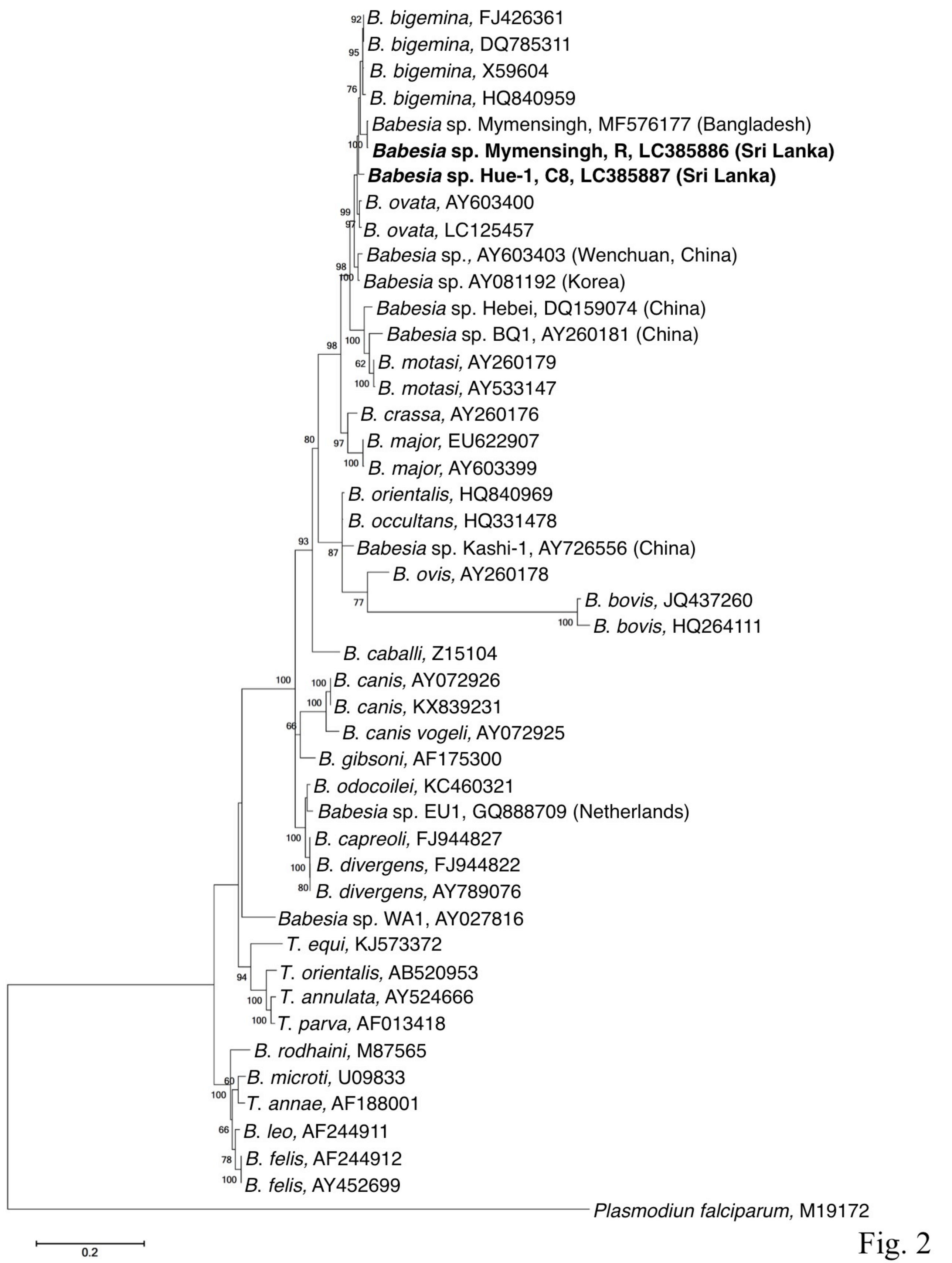
B. bigemina, FJ426361
B. bigemina, DQ785311
B. bigemina, X59604
B. bigemina, HQ840959
Babesia sp. Mymensingh, MF576177 (Bangladesh)
Babesia sp. Mymensingh, R, LC385886 (Sri Lanka)
Babesia sp. Hue-1, C8, LC385887 (Sri Lanka)
B. ovata, AY603400
B. ovata, LC125457
Babesia sp., AY603403 (Wenchuan, China)
Babesia sp. AY081192 (Korea)
Babesia sp. Hebei, DQ159074 (China)
Babesia sp. BQ1, AY260181 (China)
B. motasi, AY260179
B. motasi, AY533147
B. crassa, AY260176
B. major, EU622907
B. major, AY603399
B. orientalis, HQ840969
B. occultans, HQ331478
Babesia sp. Kashi-1, AY726556 (China)
B. ovis, AY260178
B. bovis, JQ437260
B. bovis, HQ264111
B. caballi, Z15104
B. canis, AY072926
B. canis, KX839231
B. canis vogeli, AY072925
B. gibsoni, AF175300
B. odocoilei, KC460321
Babesia sp. EU1, GQ888709 (Netherlands)
B. capreoli, FJ944827
B. divergens, FJ944822
B. divergens, AY789076
Babesia sp. WA1, AY027816
T. equi, KJ573372
T. orientalis, AB520953
T. annulata, AY524666
T. parva, AF013418
B. rodhaini, M87565
B. microti, U09833
T. annae, AF188001
B. leo, AF244911
B. felis, AF244912
B. felis, AY452699
Plasmodiun falciparum, M19172
0.2

Fig. 2

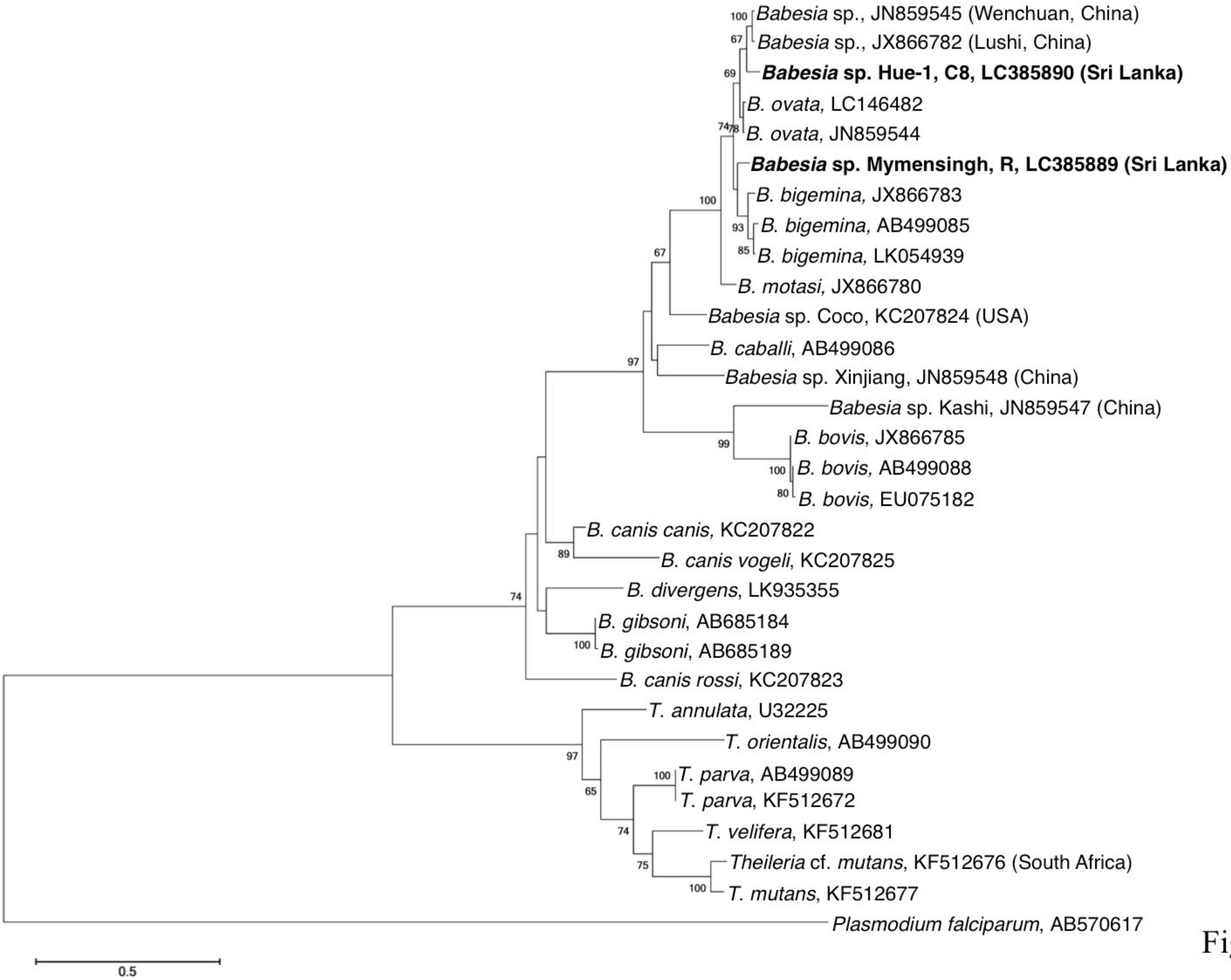
Babesia sp., JN859545 (Wenchuan, China)
Babesia sp., JX866782 (Lushi, China)
Babesia sp. Hue-1, C8, LC385890 (Sri Lanka)
B. ovata, LC146482
B. ovata, JN859544
Babesia sp. Mymensingh, R, LC385889 (Sri Lanka)
B. bigemina, JX866783
B. bigemina, AB499085
B. bigemina, LK054939
B. motasi, JX866780
Babesia sp. Coco, KC207824 (USA)
B. caballi, AB499086
Babesia sp. Xinjiang, JN859548 (China)
Babesia sp. Kashi, JN859547 (China)
B. bovis, JX866785
B. bovis, AB499088
B. bovis, EU075182
B. canis canis, KC207822
B. canis vogeli, KC207825
B. divergens, LK935355
B. gibsoni, AB685184
B. gibsoni, AB685189
B. canis rossi, KC207823
T. annulata, U32225
T. orientalis, AB499090
T. parva, AB499089
T. parva, KF512672
T. velifera, KF512681
Theileria cf. mutans, KF512676 (South Africa)
T. mutans, KF512677
Plasmodium falciparum, AB570617
100
67
69
74
78
100
93
85
67
97
99
100
80
89
74
100
97
65
100
74
75
100
0.5

Fig. 3

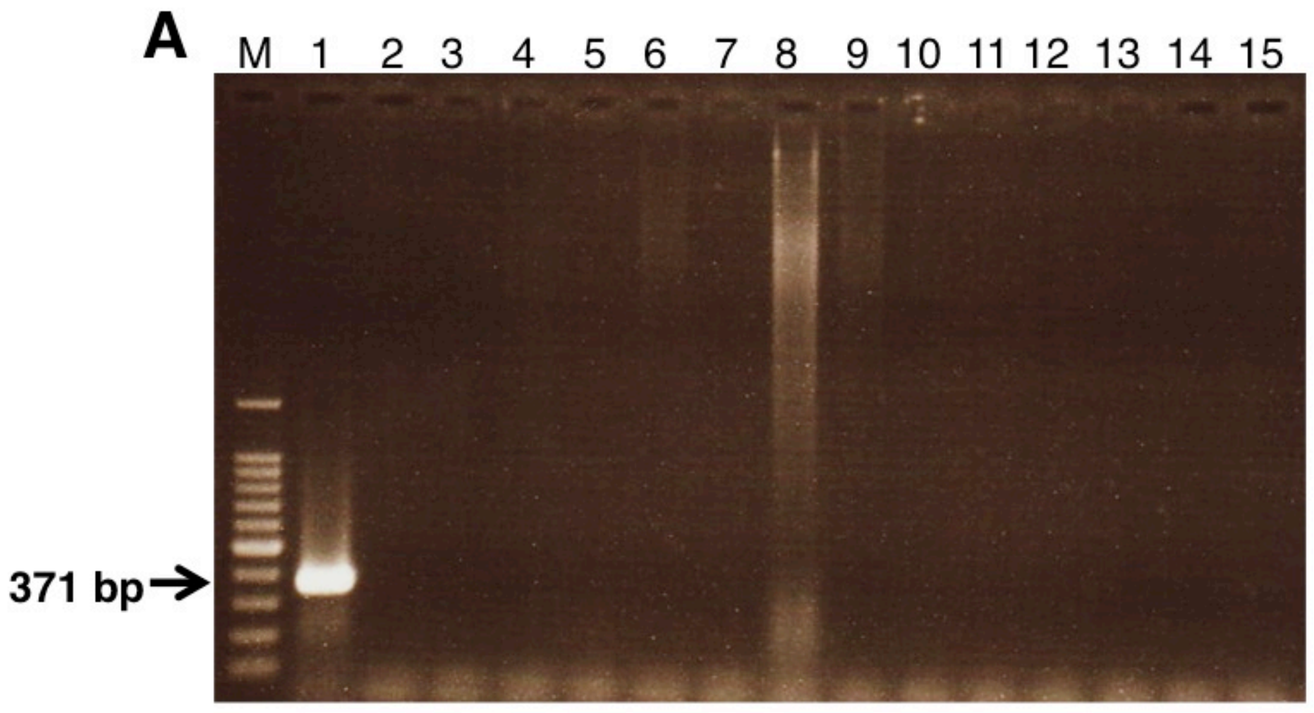
A
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
371 bp
B
M I L1 L2 R S T1 T2 V C2 C5 C6 C7 C8 NC
371 bp

Fig. 4

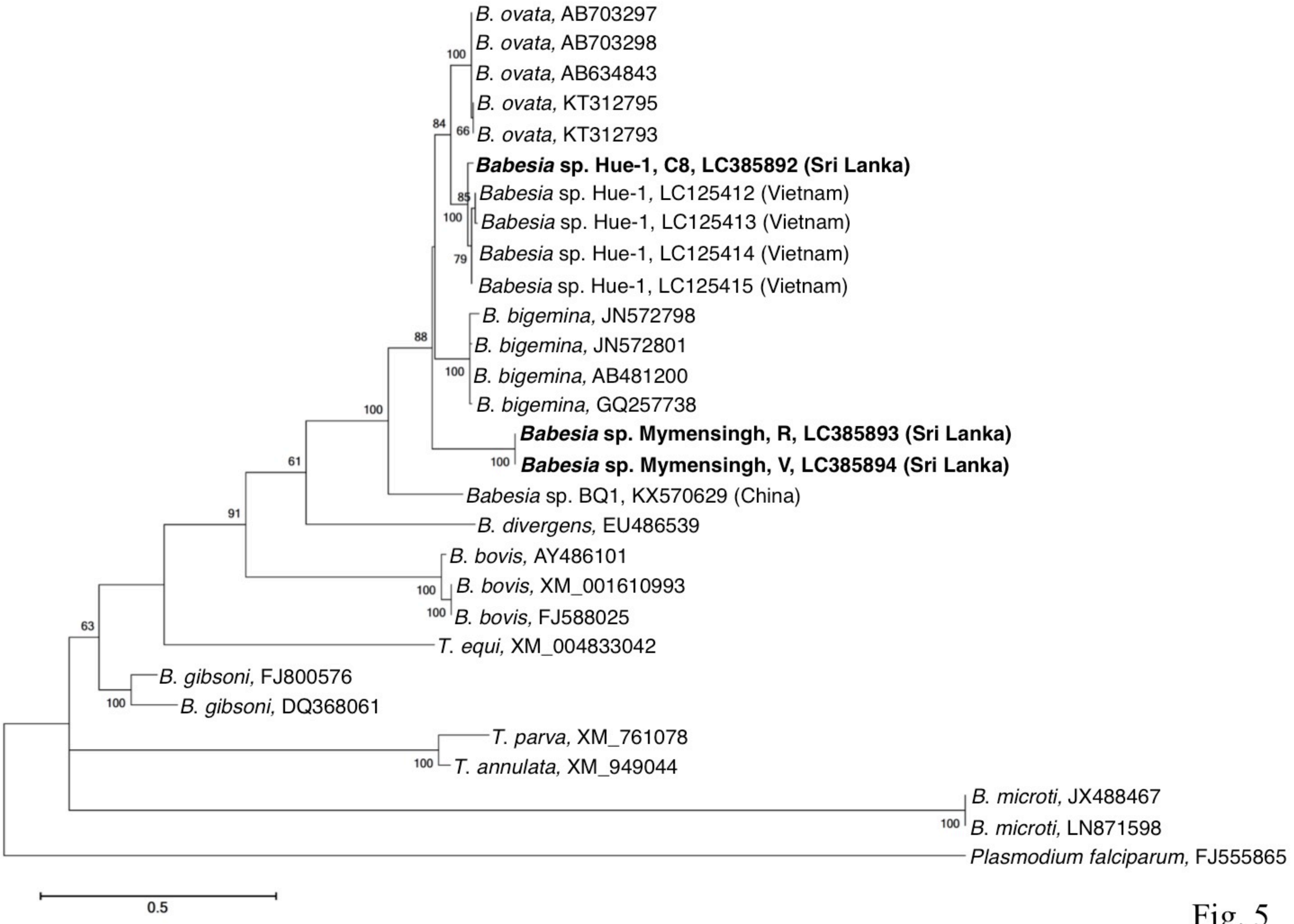
B. ovata, AB703297
B. ovata, AB703298
B. ovata, AB634843
B. ovata, KT312795
B. ovata, KT312793
Babesia sp. Hue-1, C8, LC385892 (Sri Lanka)
Babesia sp. Hue-1, LC125412 (Vietnam)
Babesia sp. Hue-1, LC125413 (Vietnam)
Babesia sp. Hue-1, LC125414 (Vietnam)
Babesia sp. Hue-1, LC125415 (Vietnam)
B. bigemina, JN572798
B. bigemina, JN572801
B. bigemina, AB481200
B. bigemina, GQ257738
Babesia sp. Mymensingh, R, LC385893 (Sri Lanka)
Babesia sp. Mymensingh, V, LC385894 (Sri Lanka)
Babesia sp. BQ1, KX570629 (China)
B. divergens, EU486539
B. bovis, AY486101
B. bovis, XM_001610993
B. bovis, FJ588025
T. equi, XM_004833042
B. gibsoni, FJ800576
B. gibsoni, DQ368061
T. parva, XM_761078
T. annulata, XM_949044
B. microti, JX488467
B. microti, LN871598
Plasmodium falciparum, FJ555865
100
84
66
85
100
79
88
100
100
61
91
100
100
63
100
100
100
0.5

Fig. 5