Tick-borne haemoparasites and Anaplasmataceae in domestic dogs in Zambia

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Abstract

Tick-borne diseases (TBDs), including emerging and re-emerging infectious diseases, are important threats to human and animal health worldwide. Indeed, the number of reported human and animal infectious cases of novel TBD agents has increased in recent decades. However, TBDs tend to be neglected, especially in resource-limited countries that often have limited diagnostic capacity. The aim of this molecular survey was to detect and characterise tick-borne pathogens (*Babesia*, *Theileria*, and *Hepatozoon* parasites and Anaplasmataceae bacteria) in domestic dogs in Zambia. In total, 247 canine peripheral blood samples were collected in Shangombo, Mazabuka, Lusaka, and Monze. Conventional PCR to detect the selected pathogens was performed using DNA extracted from canine blood. One hundred eleven samples were positive for protozoa and 5 were positive for Anaplasmataceae. Sequencing of thirty-five randomly selected protozoan-positive samples revealed the presence of *Babesia rossi*, *Babesia vogeli*, and *Hepatozoon canis* 18S rDNA. Based on these sequences, a multiplex PCR system was developed to yield PCR products with different amplicons, the size of which depended on the parasite species; thus, each species could be identified without the need for sequence analysis. Approximately 40% of dogs were positive for *H. canis*. In particular, the positive rate (75.2%) of *H. canis* infection was significantly higher in Shangombo than in other sampling sites. Multiplex PCR assay detected *B. rossi* and *B. vogeli* infections in five and seven dogs, respectively,

indicating that this approach is useful for detecting parasites with low prevalence. Sequencing analysis of *gltA* and *groEL* genes revealed that two and one dogs in Lusaka were infected with *A. platys* and *E. canis*, respectively. The data indicated that Zambian dogs were infected with multiple tick-borne pathogens such as *H. canis*, *B. rossi*, *B. vogeli*, *A. platys*, *E. canis* and uncharacterized *Ehrlichia* sp. Since some of these parasites are zoonotic, concerted efforts are needed to raise awareness of, and control, these tick-borne pathogens.

Introduction

Ticks are the second most common blood sucking arthropods next to mosquitoes. They not only cause anaemia in their animal hosts, but also carry and transmit a wide variety of viruses, bacteria, and protozoa, some of which cause tick-borne diseases (TBDs) (de la Fuente et al., 2008; Otranto et al., 2014). These TBDs not only include multiple existing infectious diseases, but also comprise emerging and re-emerging infectious diseases. An example of one such emerging TBD is severe fever with thrombocytopaenia syndrome, which was reported to be endemic to China in 2011 and which poses serious threats to human and animal health (Parola et al., 2005; Yu et al., 2011). Moreover, in the past two decades, the number of reported cases of infection with novel TBDs in humans and animals has increased (Kernif et al., 2016).

Given that some TBDs in humans are zoonoses, it is important to identify the tick-borne pathogens in pets, livestock, and wild animals and elucidate the factors that determine their prevalence. The most common tick-borne protozoan pathogens of dogs are *Babesia* and *Hepatozoon* (Homer et al., 2000; Baneth et al., 2003). These haemoparasites live in mammalian blood cells and cause severe diseases and sometimes death in infected animals (Schnittger et al., 2012). Specifically, *Babesia gibsoni*, *Babesia canis*, *Babesia rossi*, and *Babesia vogeli* are causative agents of canine babesiosis (biliary fever). *B. gibsoni* is distributed in Asia, North America, Europe, and northern and eastern Africa (Farwell et al., 1982; Jefferies et al., 2003; Lobetti, 1998). *B. canis* is transmitted by *Dermacentor reticulatus*, which is prevalent in Europe (Solano-Gallego and Baneth, 2011). The other two species, *B. rossi* and *B. vogeli*, are mainly transmitted by *Haemaphysalis leachi* and *Rhipicephalus sanguineus* sensu lato (s.l.), respectively (Apanaskevich et al., 2007; Criado-Fornelio et al., 2003). The distribution of *B. rossi* is restricted to sub-Saharan Africa, while that of *B. vogeli* is worldwide (Europe, Africa, Asia, South and North America) (Oyamada et al., 2005; Matjila et al., 2008). *Hepatozoon canis* and *Hepatozoon americanum* are the agents of canine hepatozoonoses that range from being asymptomatic with low levels of parasitaemia to a severe life-threatening illness characterised by high levels of parasitaemia, fever, anaemia, and lethargy (Baneth et al., 2000; Baneth et al., 2003). These two *Hepatozoon* species are genetically and geographically distinct (Baneth et al., 2000). *H. canis* is

98 distributed in Africa, southern Europe, the Middle East, and Asia (Baneth, 2006) and is mainly
99 transmitted by *R. sanguineus* s.l. and *Haemaphysalis longicornis* (Dantas-Torres et al., 2012;
100 Murata et al., 1995). *H. americanum* is found in the Americas and is transmitted by *Amblyomma*
101 *maculatum* (Mathew et al., 1998). Recently, new *Hepatozoon* spp. were reported in dogs and
102 wildlife in Turkey and Brazil (Aydin et al., 2015; Soares et al., 2017).

103 *Anaplasma* and *Ehrlichia* species are obligate intracellular bacteria that belong to the
104 family of Anaplasmataceae. These tick-borne pathogens infect humans and animals all over the
105 world. *Anaplasma platys* is primarily found in dogs with cyclic thrombocytopaenia (Harvey et al.,
106 1978). In addition, new *Anaplasma* species that are closely related to *A. phagocytophilum*, which
107 causes human granulocytic anaplasmosis, have been detected in canine blood (Inokuma et al.,
108 2005). *Ehrlichia canis* is the causative agent of canine ehrlichiosis, which is transmitted by *R.*
109 *sanguineus* s.l. (Groves et al., 1975; Aguiar et al., 2007). While *E. canis* was initially thought to
110 be pathogenic in canines only, it was eventually also detected in human patients with the typical
111 clinical findings of ehrlichiosis (Perez et al., 2006).

112 Only a few studies have examined haemoparasites and Anaplasmataceae in domestic
113 and wild dogs in Zambia. Baba et al. (2012) described the case of a dog that was exported from
114 Zambia to Japan and was infected with *E. canis*, while Nalubamba et al. (2011) showed that of

1,196 samples from domestic dogs in Lusaka, only 2.4% were positive for *Babesia* parasites. This is supported by Williams et al. (2014), whose molecular survey on 11 wild and eight domestic dogs in the Eastern and Western Provinces of Zambia showed that all were negative for *Babesia* infection. However, they did find that 65% of wild dogs and 13% of domestic dogs were infected with *Hepatozoon*. Recently, Vlahakis et al. (2017) described the first molecular evidence of *A. platys* in domestic dogs in Lusaka.

To better understand the infection status and distribution of tick-borne pathogens in Zambian domestic dogs, we subjected blood samples from 247 domestic dogs living in four districts of Zambia to our newly developed multiplex PCR assay, which differentiates between the main tick-borne canine haemoparasites. This molecular survey showed that some Zambian dogs are infected with *Anaplasma*, *Ehrlichia*, *Babesia*, and especially *Hepatozoon*.

Materials and methods

Ethics

All procedures were performed in accordance with the guidelines established by the Animal Experiment Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Sapporo, Japan).

Dogs

From January to May 2016, 247 peripheral blood samples were collected from privately owned dogs (149 male and 98 female) in four different locations in Zambia (Figure 1): Lusaka (15.23°S, 28.19°E) (n = 50), Mazabuka (15.51°S, 27.44°E) (n = 50), Monze (16.16°S, 27.28°E) (n = 50), and Shangombo (16.19°S, 22.06°E) (n = 97). Lusaka, Mazabuka and Monza are relatively urbanized cities/towns, while Shangombo is located in a rural area close to the border with Angola. The sampling was conducted on the randomly selected dogs, which participated in the rabies vaccination campaign. The age of the dogs ranged from three months to fifteen years with averages of thirty-nine, thirty-nine, thirty, and twenty-seven months in Lusaka, Mazabuka, Monze, and Shangombo, respectively. DNA was extracted from 200 µl of EDTA-anticoagulated whole blood using DNAzol BD Reagent (Invitrogen, Massachusetts, USA) or a QIAamp DNA Blood Mini kit (Qiagen, Tokyo, Japan) and stored at −20°C until used.

PCR

PCRs were performed by using the primers listed in Table 1. *Babesia*, *Theileria*, and/or *Hepatozoon* parasites were detected by nested PCR that amplifies a 1.4–1.6 kb fragment of the parasite's 18S rDNA: BTH 18S 1st F and BTH 18S 1st R were used for primary amplification while BTH 18S 2nd F and BTH 18S 2nd R were used for secondary amplification, as described previously (Masatani et al., 2017). Members of the Anaplasmataceae family were firstly detected using EHR16SD and EHR16SR, which amplify a 345-bp fragment of 16S ribosomal DNA (rDNA) from these bacteria (Parola et al., 2000). The positive samples were further characterized by additional PCRs targeting citrate synthase (*gltA*) and heat-shock protein (*groEL*) genes of Anaplasmataceae. All PCR reactions were conducted in a 25 µl-reaction mixture containing 12.5 µl of 2 × Gflex PCR Buffer (Mg^{2+}, dNTP plus) (TaKaRa Bio Inc., Shiga, Japan), 0.5 µl of Tks Gflex DNA Polymerase (1.25 units/µl) (TaKaRa Bio Inc.), 200 nM of each primer, and 1.0 µl of template DNA or 5-fold diluted first PCR product. The reaction conditions were 95°C for 3 min and 40 cycles of 95°C for 30 s, annealing temperature for 30 s (Table 1), and 68°C for 30 s (PCRs for Anaplasmataceae) or 90 s (PCR for *Babesia*, *Theileria*, and/or *Hepatozoon*), followed by a final extension at 68°C for 5 min. The PCR products were subjected to electrophoresis in a 1.2% agarose gel stained with Gel-Red™ (Biotium, Hayward, CA).

Multiplex PCR

The conventional nested PCR yielded many positive samples. To reduce the need for cost- and time-intensive sequencing, we first sequenced 35 randomly selected nested PCR-positive blood samples. Since we detected *B. rossi*, *B. vogeli*, and *H. canis*, a multiplex PCR that differentiated between these species was developed. Thus, the 18S rDNA sequences of the 35 samples were aligned and three forward primers that were specific for each parasite (Br_18S_F, Bv_18S_F, and Hc_18S_F) and a reverse primer that recognized the same sequence in all three parasites (BTH_multi_R) were designed (Table 1). The amplified products for *B. rossi*, *B. vogeli*, and *H. canis* comprise 522 bp, 1024 bp, and 360 bp, respectively. The multiplex PCR was conducted by using the Multiplex PCR Assay Kit (TaKaRa Bio Inc.). A 25 µl-reaction mixture that consisted of 12.5 µl of Multiplex PCR Mix 2, 0.125 µl of Multiplex PCR Mix 1, 200 nM of the four primers (Br_18S_F, Bv_18S_F, Hc_18S_F, and BTH_multi_R), and 1.0 µl of 5-fold diluted product of the PCR using the BTH 18S 1st F and BTH 18S 1st R primers was generated. The reaction conditions were 94°C for 1 min and 40 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 10 min. The parasite species were identified according to the size of the PCR amplicon in an agarose gel electrophoresis gel.

Cloning

178 To analyse parasite sequences present in samples infected with multiple parasites,
179 parasite 18S rDNA was amplified using a high-fidelity PCR enzyme, KOD-Plus-Ver.-2 DNA
180 polymerase (Toyobo, Osaka, Japan), in 25 µl of reaction mixture containing 2.5 µl of 10× Buffer
181 for KOD-Plus-Neo, 300 nM of each primer, 200 µM dNTPs, 1 mM $MgSO_4$, 0.5 unit of KOD-
182 Plus-Neo DNA polymerase, and 1.0 µl of template DNA or diluted (5×) first-round PCR product.
183 The reaction conditions were 94°C for 2 min, followed by 40 cycles of 98°C for 10 s, 55°C for
184 30 s, and 68°C for 30 s, followed by a final extension at 68°C for 2 min. The second-round PCR
185 product was A-tailed using 10× A-attachment Mix (Toyobo) and then cloned into a T-vector
186 pMD20 (TaKaRa Bio Inc.).

187 **Sequencing**

188 The PCR products were purified by using a NucleoSpin Gel and PCR Clean Up Kit
189 (Takara Bio Inc.) and sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit
190 (Applied Biosystems, Foster City, CA, USA) utilising an ABI Prism 3130x genetic analyser
191 according to the manufacturers' instructions. Only the sequences that were recovered from more
192 than three clones were considered to be genuine. The sequences that were obtained were
193 submitted to the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) under accession numbers
194 LC331056–LC331057 (18S rDNA of *B. rossi*), LC331058 (18S rDNA of *B. vogeli*), LC331059–

LC331061 (18S rDNA of *H. canis*), LC331059–LC331061 (16S rDNA of Anaplasmataceae), LC373037 and LC373038 (*gltA* of Anaplasmataceae), and LC373039-LC373041 (*groEL* of Anaplasmataceae).

Sequence data analysis

The sequences were analysed by using GENETYX version 9.1 (GENETYX Corporation, Tokyo, Japan) and were trimmed on both the 5' and 3' ends. The obtained sequences were compared with those available in public databases using nucleotide BLASTn at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was conducted by using MEGA version 6.05 (Tamura et al., 2013). ClustalW was used to align the sequences to closely related organism sequences that were deposited in the database. A neighbour-joining method was used to perform the phylogenetic analysis. Bootstrap values were obtained with 1,000 replicates.

Statistical analysis

Dogs from different regions were compared in terms of frequency of infection with specific parasites by using Fisher's exact test. For this, the Fmsb package in R 3.4.0 (R Core Team 2017) was employed. *P* values of <0.05 were considered to indicate statistical significance.

Results

Detection of protozoan parasites

Nested PCR targeting *Babesia*, *Theileria*, and *Hepatozoon* parasites was positive in 111 dogs: fourteen (28.0%) in Lusaka, eight (16.0%) in Mazurka, sixteen (32.0%) in Monze, and seventy-three (75.2%) in Shangombo (Table 2). Initially, thirty-five samples were randomly selected and the second-round PCR products from these samples were subjected to sequence analysis. The resulting data indicated the presence of *B. rossi*, *B. vogeli*, and *H. canis*. To distinguish between these three parasites, we developed a multiplex PCR assay based on species-specific primers (Table 1). Since the PCR band of each parasite differs in size, the species can be identified without having to perform sequence analysis. A representative electrophoresis of the multiplex PCR products is shown in Figure 2.

Multiplex PCR assay of all 111 BTH-PCR-positive samples showed that five dogs were infected with *B. rossi*. Four dogs were from Mazabuka and one from Shangombo. None of the samples from Lusaka and Monze was positive for *B. rossi*. Thus, the *B. rossi* infection rates in Lusaka, Mazabuka, Monze, and Shangombo were 0%, 8.0%, 0%, and 1.0%, respectively.

227 The multiplex PCR also showed that seven samples were positive for *B. vogeli*. One, four,
228 and two *B. vogeli* infections were found in dogs from Lusaka, Mazabuka, and Monze,
229 respectively. None of the samples from Shangombo were positive for *B. vogeli*. Thus, the *B.*
230 *vogeli* infection rates in Lusaka, Mazabuka, Monze, and Shangombo were 2.0%, 8.0%, 4.0%,
231 and 0%, respectively.

232 The multiplex PCR also showed that 100 dogs were infected with *H. canis* (Table 2).
233 None of the samples from Mazabuka were positive for *H. canis*. The positive rate of *H. canis* in
234 Shangombo (75.2%) was significantly higher than that in Lusaka (26.0%) or Monze (28.0%)
235 (both $p<0.05$). One dog from Shangombo was infected with both *H. canis* and *B. rossi*. The
236 presence of the two parasites was confirmed by cloning and sequencing of the 18S rDNA PCR
237 products (data not shown).

238 Sequencing analysis of the 18S rDNA PCR products of the five *B. rossi* infections
239 yielded two sequence types. One (BR1) showed 99.9% (1,428/1,430 bp) nucleotide identity with
240 *B. rossi* detected in black-backed jackals (*Canis mesomelas*) in South Africa (GenBank
241 accession no.: KY463434). The other (BR2) showed 100% (1430/1430 bp) nucleotide identity
242 with *B. rossi* from domestic dogs in Nigeria (GenBank accession no.: AB935165).

Sequencing analysis of the 18S rDNA PCR products of the seven *B. vogeli* infections yielded a single sequence type (BV1) that bore 100% (1429/1429 bp) nucleotide identity with *B. vogeli* detected in China, Japan, and Brazil (GenBank accession nos.: HM590440, AY077719, and AY371196, respectively).

Sequence analysis of the 18S rDNA PCR products from *H. canis* infections revealed four different sequence types. The most common type (HC1, 77.3% of all sequenced *H. canis*-positive samples) harboured two base pair mismatches (1,528/1,530 bp identity) with respect to *H. canis* sequences reported in dogs in Sudan (GenBank accession no.: DQ111754). Two other sequences (HC2 and HC3) exhibited 99.7% (1,526/1,530 bp) and 99.9% (1,529/1,530 bp) nucleotide identity with *H. canis* from a golden jackal (*Canis aureus*) in Eurasia (GenBank accession no.: KX712126 and KX712127).

The sequence types detected in this study are summarized in Table 3. Phylogenetic analysis showed that sequence types HC1–HC4 were located in the same cluster with *H. canis,* that sequence types BR1 and BR2 clustered together with *B. rossi*, and that the BV1 sequence type was located in a cluster of *B. vogeli* (Figure 3).

Detection of Anaplasmataceae

259 Of the 247 dogs, five (three from Lusaka and two from Shangombo) tested positive for
260 Anaplasmataceae by 16S rDNA PCR (Table 2). Sequencing analysis of the amplified products
261 from these five dogs identified three different sequences (EHR1-3). EHR1 was detected in a dog
262 from Lusaka (LuF11) and exhibited 98.4% (300/305 bp) nucleotide identity with *E. canis* and
263 several uncharacterized *Ehrlichia* spp. EHR2 was detected in the two infected dogs from
264 Shangombo (ZD#55 and ZD#56) and showed 99.7% (304/305 bp) nucleotide identity with
265 several uncharacterized *Ehrlichia* spp. EHR3 was detected in two dogs from Lusaka (LuF9 and
266 LuF24) and showed 100% (305/305 bp) nucleotide identity with the *A. platys* sequence found in
267 *R. sanguineus* s.l. ticks that had been collected from dogs in the Democratic Republic of the
268 Congo (DRC) (GenBank accession no.: AF478131).

269 In order to further characterize Anaplasmataceae, PCRs amplifying *gltA* and *groEL* were
270 carried out using several primer sets shown in Table 1. Three samples (LuF9, LuF24, and
271 LuF11) were successfully amplified, while two samples (ZD#55 and ZD#56) having identical
272 16S rDNA sequence were not amplified.

273 Sequencing analysis of the amplified products identified two and three different
274 sequences of *gltA* and *groEL*, respectively. One *gltA* sequence (GLTA1) detected from LuF11
275 showed 100% (1,033/1,033 bp) identity with *E. canis* strain YZ-1 reported from Yangzhou,

China (GenBank accession no.: CP025749). The other *gltA* sequence (GLTA2) detected from LuF9 and LuF24 showed 99.7% (1,048/1,051 bp) identity with *A. platys* found in *R. sanguineus* s.l. collected from a dog in the DRC (GenBank accession no.: AF478130). Out of three *groEL* sequences (GROEL1-3), GROEL1 detected from LuF11 showed 100% (1,060/1,060 bp) identity with *E. canis* strain YZ-1. GROEL2 detected from LuF9 and GROEL3 detected from LuF24 showed 99.7% (1,072/1,075 bp) and 99.8% (1,073/1,075 bp) identities with *A. platys* found in *R. sanguineus* s.l. ticks collected from a dog in the DRC (GenBank accession no.: AF478129), respectively.

Phylogenetic trees based on *gltA* and *groEL* genes are shown in Figures 4A and 4B, respectively. The sequences obtained from LuF11 were clustered with *E. canis* in both trees. The sequences obtained from LuF9 and LuF24 were located in the same cluster with *A. platys* in both trees.

Discussion

The aim of this study was to detect and characterise haemoparasites and Anaplasmataceae in domestic dogs in Zambia, where TBDs tend to be overlooked due to limited diagnostic capacity. Here, we provide molecular evidence showing that Zambian domestic dogs

293 are infected with multiple tick-borne pathogens, namely, *H. canis*, *B. rossi*, and *B. vogeli*, *A.*
294 *platys*, *E. canis* and uncharacterized *Ehrlichia* sp.

295 We detected *H. canis* infections in three of the four sampled locations (Mazabuka was
296 the exception) (Table 2). The dogs from Monze and Lusaka have similar frequencies of *H. canis*
297 infection (28.0% and 26.0%, respectively). By contrast, three-quarters of the dogs from
298 Shangombo were infected with *H. canis*. This is remarkable given that other studies of dogs in
299 Zambia, Angola, and Sudan showed 12.6%, 17.5% and 42.3% *H. canis*-positivity rates,
300 respectively (Williams et al., 2014; Cardoso et al., 2016; Oyamada et al., 2005). It may be that
301 there are more opportunities for dogs to be exposed to ticks in Shangombo for example due to
302 the differences in dog management styles between urban and rural areas in Zambia. Alternatively,
303 or in addition, the ticks in Shangombo may be more susceptible to infection with *H. canis* than
304 the ticks in other districts. It should be mentioned that there was no significant difference in age
305 between *H. canis*-infected and non-infected dogs in Shangombo (the average age were 26.7 and
306 29.5 months, respectively) (Mann-Whitney U test, $P = 0.77$). Future analyses of the involved tick
307 vectors in Shangombo will help to understand the high endemicity of *H. canis* in this area.

308 Given that we found a large number of canine blood samples were positive for the
309 nested PCR that detects *Babesia*, *Theileria*, and/or *Hepatozoon*, and sequencing of thirty-five

310 randomly selected positive samples indicated the presence of *B. vogeli*, *B. rossi*, and *H. canis*, we
311 developed a multiplex PCR assay that easily distinguishes these three species from each other on
312 the basis of their PCR band sizes. This approach is useful when there are so many nested PCR-
313 positive samples that it becomes impractical in terms of cost and time to sequence them all. This
314 is particularly true when the positive samples are dominated by a single parasite, such as *H. canis*
315 in the present case. If we had followed the usual approach of sequencing a handful of randomly
316 selected nested PCR samples, we may have missed the *Babesia* infections, which were only
317 present in 10% of the nested PCR-positive samples. Indeed, despite the high frequency of *H.*
318 *canis*, our multiplex PCR assay detected five *B. rossi*-infected and seven *B. vogeli*-infected
319 samples. It is not clear why the dogs had a low rate of *Babesia* infections and a high rate of *H.*
320 *canis* infections. This is primarily because very little is known about the life cycles, transmission
321 routes, and pathogenic potential of these different parasites.

322 One dog in Shangombo was co-infected with *H. canis* and *B. rossi*. Several other
323 studies report co-infection of *H. canis* and *Babesia* spp. in dogs (Cardoso et al., 2010; Rojas et al.,
324 2014). Such co-infections are partly responsible for the variable pathogenicity of these parasites
325 in dogs (Munson et al., 2008; Sasanelli et al., 2009). Veterinarians should be aware of the
326 possibility of such parasite co-infections when a dog presents with a parasite-related illness that
327 is unusually severe or is accompanied by uncharacteristic symptoms.

328 The present study detected *A. platys* infections in two female dogs in Lusaka. The
329 presence of *A. platys* in dogs has been reported elsewhere, including in Africa (Carvalho et al.,
330 2017; Inokuma et al., 2002; Beugnet and Marié, 2009; Oya,mada et al., 2005). *A. platys* infects
331 the platelets of dogs and causes infectious canine cyclic thrombocytopaenia. It is usually a mild
332 or asymptomatic disease. In general, *A. platys* is transmitted by the *R. sanguineus* s.l. tick. A
333 recent study suggested that *A. platys* can also be transmitted vertically from infected bitches to
334 their puppies, either transplacentally or during the perinatal period (Matei et al., 2017). Human
335 cases of *A. platys* have been reported: two women in Venezuela (Arraga-Alvarado et al., 2014)
336 and one veterinary professional in South Africa (Maggi et al., 2013) were found to be infected
337 with *A. platys*. Thus, dog owners and veterinarians in these regions should be aware of the risk of
338 *A. platys* infection in humans.

339 *Ehrlichia* infections were suspected in three dogs by sequencing analysis of
340 Anaplasmataceae-specific 16S rDNA PCR. Additional PCRs targeting *gltA* and *groEL* of
341 Anaplasmataceae were only successful in one dog from Lusaka, which was shown to be infected
342 with *E. canis*. However, the PCR assays did not yield any amplicons from two *Ehrlichia*-positive
343 dogs in Shangombo. Although several other sets of primers were employed to amplify these
344 genes in the two dogs, none of the PCRs were successful (data not shown). These results may
345 indicate that the dogs in Shangombo were infected with uncharacterized *Ehrlichia* sp. which are

346 genetically distinct form those previously reported. To better understand the genetic diversity of
347 *Ehrlichia* sp., further studies on the *Ehrlichia* spp. in Zambian dogs are warranted.

348 **5. Conclusions**

349 This study shows that Zambian dogs are infected with several pathogens, namely, *H.*
350 *canis, B. rossi, B. vogeli, A. platys, E. canis* and uncharacterized *Ehrlichia* sp. Our species-
351 discriminating multiplex PCR is useful for screening for canine haemoparasites, even when one
352 parasite is vastly predominant.

353

354 **Competing interests**

355 The authors declare no competing interests.

356

357 **Contributions**

358 R.N. and Y.Q. conceived and designed the experiments; Y.Q., C.K., R.N., M.K., S,
359 E.S., W.M., M.B.H., and M.S. collected samples; Y.Q., M.J.T., and R.N. performed the
360 experiments; Y.Q., C.K., and R.N. analysed the data; Y.Q. and R.N. wrote the paper; K.K., A.T.,
361 H.S., and R.N. edited and approved the manuscript.

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522

523

524 **Figure annotations**

525

526 **Figure 1. Dog sample collection sites in Zambia.**

527

528 **Figure 2. A representative electrophoresis gel of the multiplex PCR products.** M: marker;
529 HC: *Hepatozoon canis*; BR: *Babesia rossi*; BV: *Babesia vogeli*; NC: negative control.

530

531 **Figure 3. Phylogenetic analysis of the *Babesia* spp. and *Hepatozoon* spp. detected in canine**
532 **blood.** The analysis based on the almost full-length sequences of 18S rDNA was conducted
533 by using a Neighbour joining method. The tree is rooted with *Plasmodium falciparum*. All
534 bootstrap values from 1,000 replications are shown on the interior branch nodes. The
535 sequences obtained in this study are indicated in red.

536 **Figure 4. Phylogenetic analyses of the Anaplasmataceae detected in canine blood.** The
537 analyses based on partial sequences of (A) *gltA* and (B) *groEL* were conducted by using a
538 Neighbour-joining method. The trees are rooted with *Rickettsia rickettsii*. All bootstrap

539 values from 1,000 replications are shown on the interior branch nodes. The sequences

540 obtained in this study are indicated in red.

Table 1. PCR primers amplifying haemoparasites and Anaplasmataceae.

Primer name	Sequence (5' to 3')	Target (PCR type)	Annealing temperature (°C)	Reference
EHR16SD	GGTACCYACAGAAGAAGTCC	16S rDNA of Anaplasmataceae (single PCR)	55	Parola et al., 2000
EHR16SR	TAGCACTCATCGTTTACAGC			
CS7F2	ATGRTAGAAAAWGCTGTTTT	*gltA* of *A. platys* (single PCR)	55	Ybañez et al., 2012
CS1033R	GCAAAGAATGCRGTAKACAT			
EHRCS-131F	CAGGATTTATGTCTACTGCTGCTTG	*gltA* of Anaplasmataceae (1st PCR)	50	Loftis et al., 2015
EHRCS-1226R	CCAGTATATAAYTGACGWGGACG			
EHRCS-131F	CAGGATTTATGTCTACTGCTGCTTG	*gltA* of Anaplasmataceae (2nd PCR)	50	Loftis et al., 2015
EHRCS-879R	TIGCKCCACCATGAGCTG			
EHRCS-754F	ATGCTGATCATGARCAAAATG	*gltA* of Anaplasmataceae (2nd PCR)	50	Loftis et al., 2015
EHRCS-1226R	CCAGTATATAAYTGACGWGGACG			
HS1-F	CGYCAGTGGGCTGGTAATGAA	*groEL* of Anaplasmataceae (1st PCR)	54	Sumner et al., 1997
HS6-R	CCWCCWGGTACWACACCTTC			
HS3-F	ATAGTYATGAAGGAGAGTGAT	*groEL* of *Anaplasma* (2nd PCR)	50	Liz et al., 2000
HSV-R	TCAACAGCAGCTCTAGTWG			
groEL-fwd3	TGGCAAATGTAGTTGTAACAGG	*groEL* of *Ehrlichia* (2nd PCR)	50	Gofton et al., 2016
groEL-rev2	GCCGACTTTTAGTACAGCAA			
BTH 18S 1st F	GTGAAACTGCGAATGGCTCATTAC	18S rDNA of *Babesia*, *Theileria* and *Hepatozoon* parasites (1st PCR)	55	Masatani et al., 2017
BTH 18S 1st R	AAGTGATAAGGTTCACAAAACTTCCC			
BTH 18S 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG	18S rDNA of *Babesia*, *Theileria* and *Hepatozoon* parasites (2nd PCR)	55	Masatani et al., 2017
BTH 18S 2nd R	CGGTCCGAATAATTCACCGGAT			
Br_18S_F	GTATTTTTGCTTGGCGGTTT	18S rDNA of *B. rossi* (Multiplex PCR)	55	This study
Bv_18S_F	TTAGTTTGAAACCCGCCTTG	18S rDNA of *B. vogeli* (Multiplex PCR)		This study
Hc_18S_F	TAAGAGCTAAATTAATGATTGATAGGG	18S rDNA of *H. canis* (Multiplex PCR)		This study
BTH_multi_R	CCCGTGTTGAGTCAAATTAAGC	18S rDNA of *B. vogeli*, *B. vogeli*, and *H. canis* (Multiplex PCR)		This study

Table 2. Detection of Anaplasmataceae, *Babesia rossi*, *Babesia vogeli*, and *Hepatozoon canis* by PCR and species-discriminating multiplex PCR.

					Identified species[a]		
Location	Sex	Total no.	Anaplasmataceae PCR positive	BTH PCR positive	*Babesia rossi*	*Babesia vogeli*	*Hepatozoon canis*
Lusaka	Male	25	0	8	0	1 (1)	7 (2)
	Female	25	3	6	0	0	6 (1)
Mazabuka	Male	25	0	1	1 (1)	0	0
	Female	25	0	7	3 (3)	4 (4)	0
Monze	Male	25	0	11	0	1 (1)	10 (2)
	Female	25	0	5	0	1	4 (2)
Shangombo	Male	74	2	56	1[b]	0	56 (10)[b]
	Female	23	0	17	0	0	17 (5)

[a]Number in brackets indicates the number of samples confirmed by sequencing analysis of BTH PCR products.

[b]One dog was positive for both *Babesia rossi* and *Hepatozoon canis.*

Table 3. Summary of sequence types detected for *Babesia rossi*, *Babesia vogeli*, and *Hepatozoon canis* from each sampling site.

		Location				
Parasite	Sequence type	Lusaka	Mazabuka	Monze	Shangombo	Total
Babesia rossi	BR1	0	2	0	0	2
	BR2	0	2	0	0	2
Babesia vogeli	BV1	1	1	4	0	6
Hepatozoon canis	HC1	0	0	2	15	17
	HC2	3	0	0	0	3
	HC3	0	0	1	0	1
	HC4	0	0	1	0	1

Figure 1

Figure 2

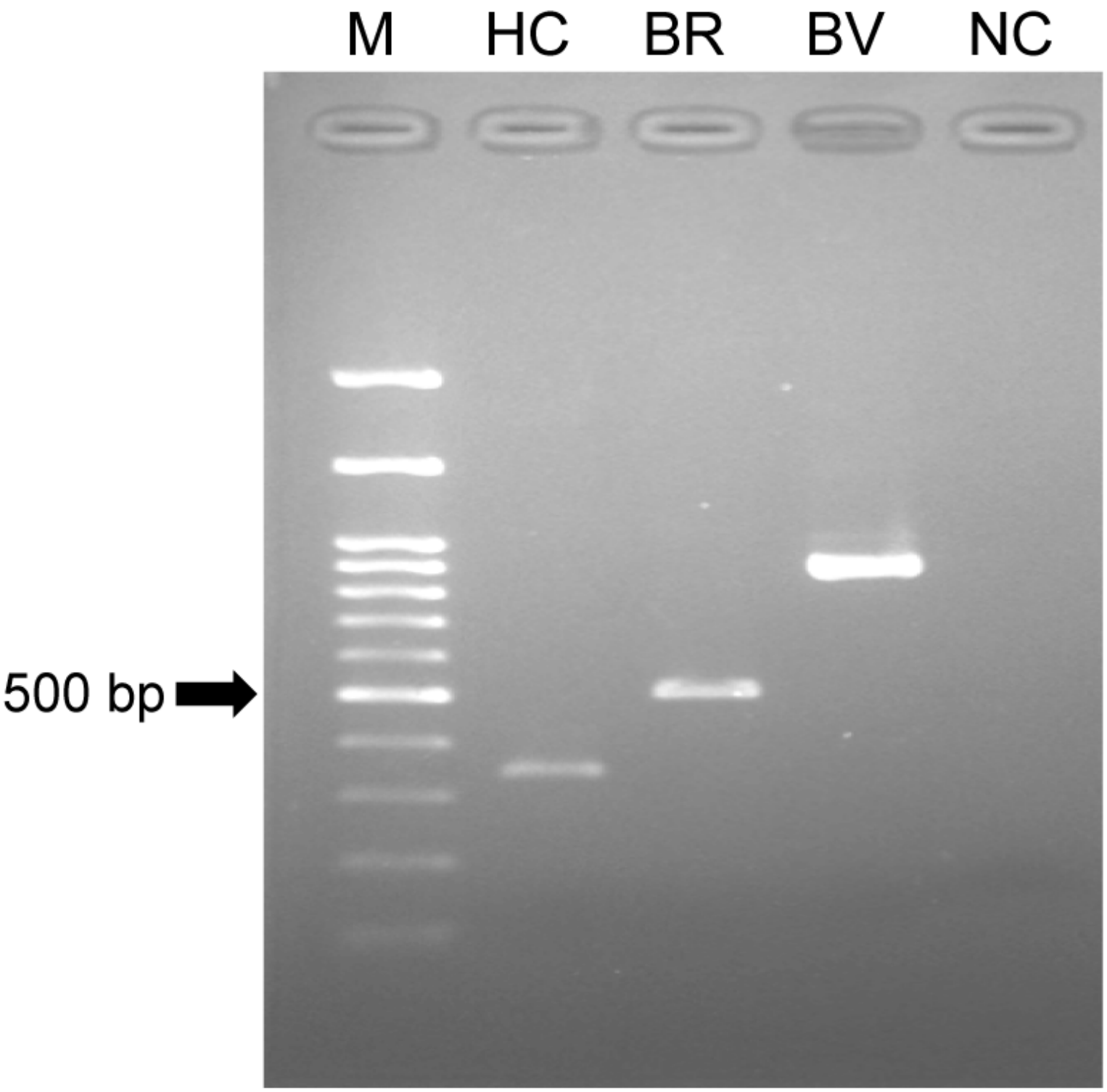


Figure 3

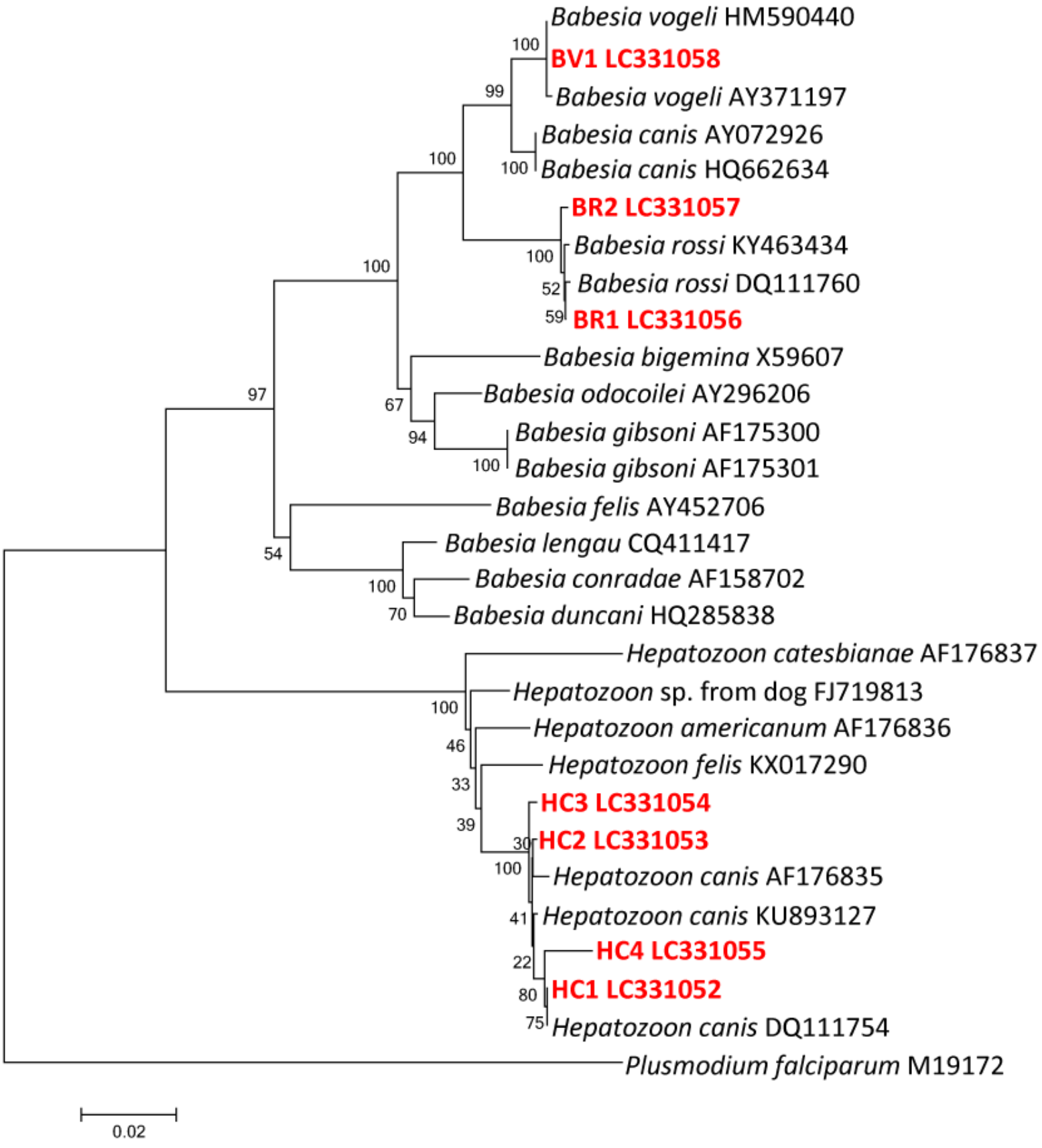
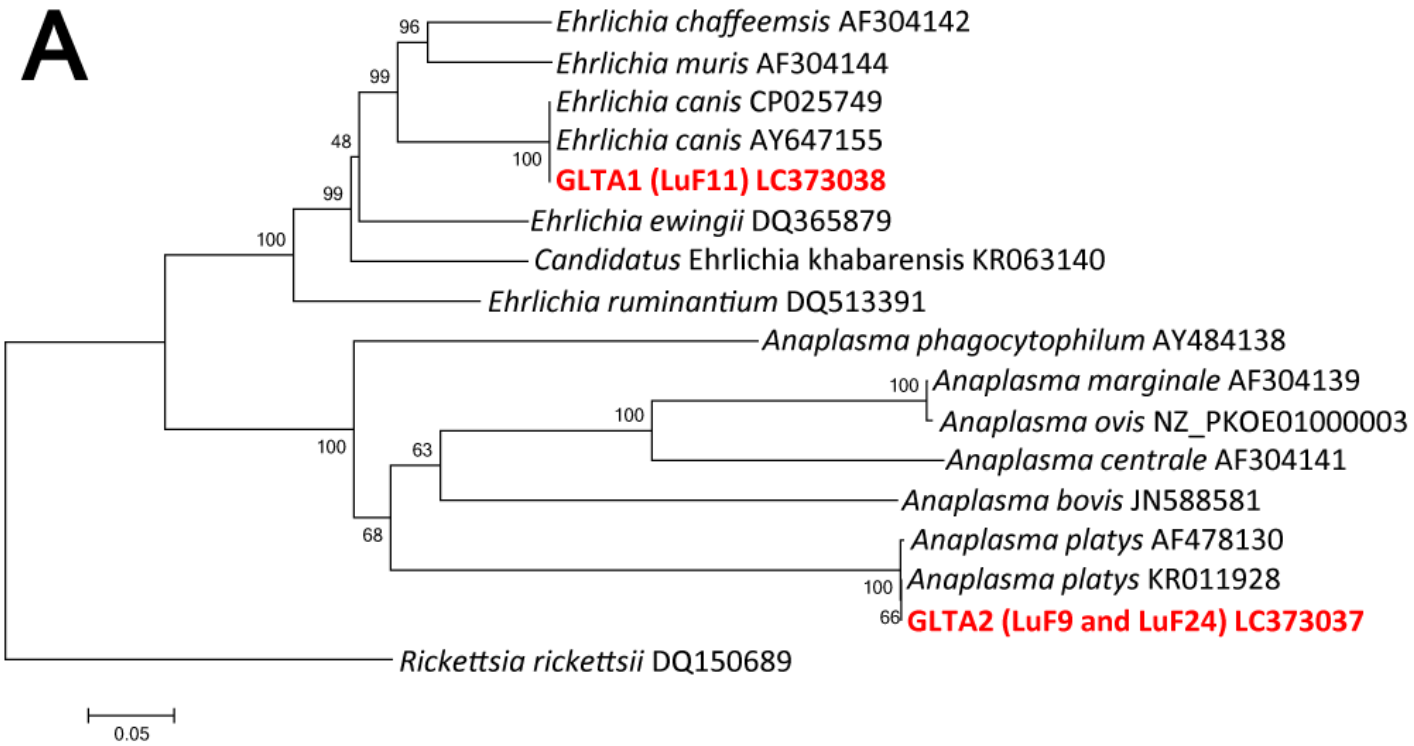
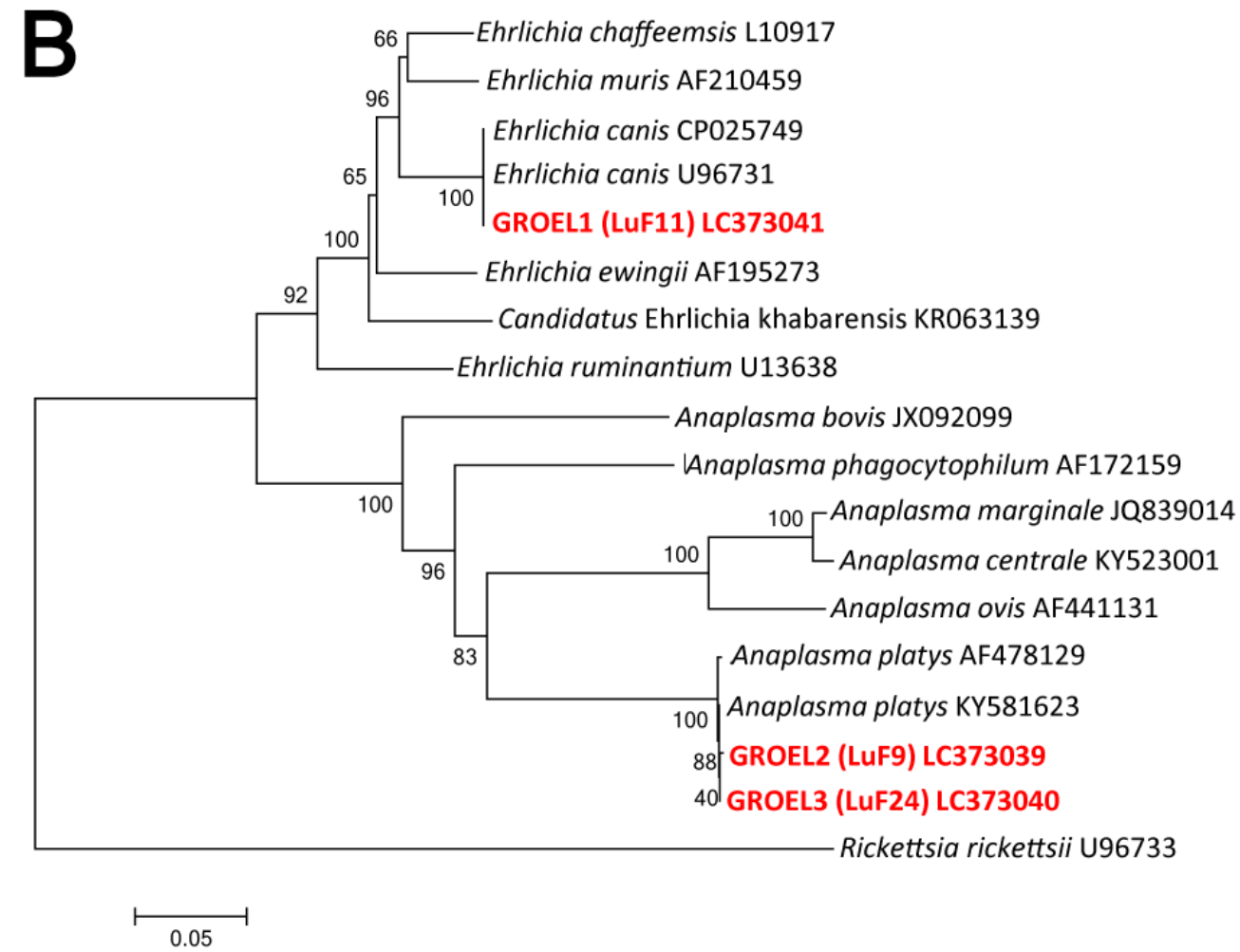

Babesia vogeli HM590440
BV1 LC331058
Babesia vogeli AY371197
Babesia canis AY072926
Babesia canis HQ662634
BR2 LC331057
Babesia rossi KY463434
Babesia rossi DQ111760
BR1 LC331056
Babesia bigemina X59607
Babesia odocoilei AY296206
Babesia gibsoni AF175300
Babesia gibsoni AF175301
Babesia felis AY452706
Babesia lengau CQ411417
Babesia conradae AF158702
Babesia duncani HQ285838
Hepatozoon catesbianae AF176837
Hepatozoon sp. from dog FJ719813
Hepatozoon americanum AF176836
Hepatozoon felis KX017290
HC3 LC331054
HC2 LC331053
Hepatozoon canis AF176835
Hepatozoon canis KU893127
HC4 LC331055
HC1 LC331052
Hepatozoon canis DQ111754
Plusmodium falciparum M19172
100
99
100
100
100
52
59
100
97
67
94
100
54
100
70
100
46
33
39
30
100
41
22
80
75
0.02

Figure 4


A
Ehrlichia chaffeemsis AF304142
Ehrlichia muris AF304144
Ehrlichia canis CP025749
Ehrlichia canis AY647155
GLTA1 (LuF11) LC373038
Ehrlichia ewingii DQ365879
Candidatus Ehrlichia khabarensis KR063140
Ehrlichia ruminantium DQ513391
Anaplasma phagocytophilum AY484138
Anaplasma marginale AF304139
Anaplasma ovis NZ_PKOE01000003
Anaplasma centrale AF304141
Anaplasma bovis JN588581
Anaplasma platys AF478130
Anaplasma platys KR011928
GLTA2 (LuF9 and LuF24) LC373037
Rickettsia rickettsii DQ150689
96
99
48
100
99
100
100
100
63
68
100
66
0.05


B
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Ehrlichia muris AF210459
Ehrlichia canis CP025749
Ehrlichia canis U96731
GROEL1 (LuF11) LC373041
Ehrlichia ewingii AF195273
Candidatus Ehrlichia khabarensis KR063139
Ehrlichia ruminantium U13638
Anaplasma bovis JX092099
Anaplasma phagocytophilum AF172159
Anaplasma marginale JQ839014
Anaplasma centrale KY523001
Anaplasma ovis AF441131
Anaplasma platys AF478129
Anaplasma platys KY581623
GROEL2 (LuF9) LC373039
GROEL3 (LuF24) LC373040
Rickettsia rickettsii U96733
66
96
65
100
100
92
100
100
100
96
83
100
88
40
0.05