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## Great diversity of Piroplasmida in Equidae in Africa and Europe, including potential new species

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1 **Great diversity of *Piroplasmida* in *Equidae* in Africa and Europe, including**  
2 **potential new species**

3  
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23 **Highlights**

- 24       • New PCR assays were developed to potentially identify all piroplasms.
- 25       • Epidemiological study was conducted on *Equidae* from sub-Saharan Africa and  
26       France.
- 27       • Four species of piroplasms infecting horse, including two potentially new ones, were  
28       detected.
- 29       • The newly identified species were characterized by amplifying three genes.

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31        **Abstract**

32        Piroplasms are *Apicomplexa* tick-borne parasites distributed worldwide. They are  
33 responsible for piroplasmosis (theileriosis and babesiosis) in Vertebrata and are therefore of  
34 medical and economic importance.

35        Herein, we developed a new real time PCR assay targeting the 5.8S rRNA gene and  
36 three standard PCR assays, targeting 18S rRNA, 28S rRNA, and *coxI* genes, for the detection  
37 of piroplasmids. These assays were first optimized and screened for specificity and  
38 sensitivity. Then, they were used to study a total of 548 blood samples and 97 ticks collected  
39 from *Equidae* in four sub-Saharan countries (Senegal, Democratic Republic of the Congo,  
40 Chad, and Djibouti) and France (Marseille and Corsica).

41        DNA of piroplasms was detected in 162 of 548 (29.5%) blood samples and in 9 of 97  
42 (9.3%) ticks. The highest prevalence in blood samples was observed in Chad in 2016 with  
43 72.9% positivity rate. Sequencing allowed the identification of four species of piroplasms,  
44 including two potentials new species. *Theileria equi* was mainly found. The highest  
45 prevalence was observed in Senegal (14 positive out of 23, 60.87%). *Babesia caballi* was  
46 detected in one horse in Senegal. Two new potential *Theileria* species were detected:  
47 *Theileria* sp. "*Africa*", observed in all areas excepted in Marseille and *Theileria* sp. "*Europa*",  
48 observed in Marseille and Corsica.

49        In conclusion, sensitive and specific PCR assays were developed for epidemiological  
50 studies of *Piroplasmida*. The circulation of multiple species of piroplasms, including two  
51 potentials new species, observed among *Equidae* from sub-Saharan Africa and France.

52

53        **Key words:** Piroplasmosis, *Equidae*, PCR assays, Sub-Saharan Africa, France.

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## 55 **1. Introduction**

56 *Apicomplexa* protists are stealth invaders, they can escape the immune response in host  
57 cells while using them as a source of nutrients (Striepen et al., 2007). Almost all  
58 apicomplexans are parasites, including multiple pathogenic species, both for humans and  
59 animals like malaria, toxoplasmosis, cryptosporidiosis, and piroplasmosis.

60 The parasites belonging to the apicomplexan order *Piroplasmida* include three genera  
61 namely: *Babesia*, *Theileria*, and *Cytauxzoon* (Megan E. Schreeg et al., 2016). Equine  
62 piroplasmosis is an infectious tick-borne disease caused by the hemoprotozoan parasites  
63 *Theileria equi* and *Babesia caballi* (Wise et al., 2013). These piroplasms affect equid species,  
64 including horses, donkeys, mules and zebras (Wise et al., 2013). *T. equi* was initially named  
65 *Piroplasma equi* by Laveran in 1901, but after the discovery of schizogony in horse  
66 lymphocytes, which is known for *Theileria* but not for *Babesia*, this parasite was reclassified  
67 by Mehlhorn and Schein as *T. equi* (Uilenberg, 2006). In 1912, *B. caballi*, formerly named  
68 *Piroplasma caballi*, was first identified as another parasite infecting equids and different from  
69 *T. equi* and was assigned to the genus *Babesia* (Nuttall and Strickland, 1912).

70 Equine piroplasmosis caused by *B. caballi* and *T. equi* is endemic in tropical and  
71 subtropical zones; the latter is most prevalent (Friedhoff and Soulé, 1996). Southern Europe  
72 and Africa are highly endemic (Wise et al., 2014). Moreover, a high genetic diversity of these  
73 parasites has been observed in Tunisia (Ros-García et al., 2013) and Jordan (QABLAN et al.,  
74 2013).

75 Equine piroplasmosis has serious impacts on horses' health. Agricultural production is  
76 strongly affected with high cost of control rules and impact on the carriage of merchandises  
77 and international commerce (World Organization for Animal Health. Biological Standards  
78 Commission., 2012). In the United States, with a population of about 9.2 million horses, the  
79 direct economic impact of the equine industry is about 39 billion dollars per year. This  
80 industry also supports approximately 1.4 million full-time jobs (The American Horse Council,

81 2005). It took 25 years and \$23 million for Southern California to eradicate equine  
82 piroplasmosis (USDA, 2010).

83 For a long time, *T. equi* and *B. caballi* were considered specific to their hosts. However,  
84 both parasites were recently identified in clinically healthy dromedaries by PCR, in Jordan  
85 (Qablan et al., 2012). Both were also detected in a dog in Croatia in 2009(Beck et al., 2009).  
86 In France, in 2010, Fritz *et al.* analyzed 166 dogs; 31 were infected by *T. equi* and one by *B.*  
87 *caballi* (Fritz, 2010). Finally, *Babesia canis*, *Babesia rossi* and *Babesia capreoli* were  
88 recently reported to infect also horses (Fritz, 2010 ;Zanet et al., 2017).

89 Piroplasmosis is a typical zoonotic vector-borne infection (Schnittger et al., 2012).  
90 However, *T. equi* and *B. caballi* are not considered to infect humans (Maslin et al., 2004).  
91 Ticks and iatrogenic blood transfers are efficient modes of transmission in equids (Ueti et al.,  
92 2005). More than 21 tick species, mainly hard ticks, are associated with the transmission of  
93 these parasites. Co-infections are frequently reported in *Equidae*, often associated with co-  
94 infestation by tick species of the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus*,  
95 (Tamzali, 2013). *Babesia* and *Theileria* exhibit different ecological relationships with their  
96 vectors and hosts. The reservoirs of *Babesia* include chronically infected animals and ticks  
97 (Yabsley and Shock, 2013). Infected ticks are able to transovarial and transstadial  
98 transmission of *B. caballi* from female ticks to its offspring. In contrast, *Theileria* are only  
99 transmitted transtadially (Ueti et al., 2005). Besides, *Equidae* are the primary reservoirs of  
100 *Theileria*.

101 Clinical signs of equine piroplasmosis are similar for both parasites (Tamzali,  
102 2013).They occur after transmission within 10 to 30 days for *B. caballi* and 12 to 19 days for  
103 *T. equi*(de Waal, 1992). In endemic areas, most of infected horses are asymptomatic carriers  
104 with low level of parasitaemia. In case of concurrent disease or stress, they may develop  
105 clinical equine piroplasmosis (Allsopp et al., 2007). Acute forms of equine piroplasmosis can

106 include fever (over 40°C), sweating, congested mucous membranes, limb and supraorbital  
107 edema, icterus, anorexia, tachypnoea, tachycardia, anemia, and occasionally petechiae or  
108 ecchymoses (Tamzali, 2013). Death may occur in severe cases.

109 In this study, our aim was to develop sensitive and specific molecular tools able to  
110 potentially detect all piroplasm species and to analyze their phylogeny, in order to perform an  
111 epidemiological study on *Equidae* from sub-Saharan Africa and France.

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## 132 2. Materials and methods

### 133 2.1 Study area and samples collection

134 In total, 548 blood samples and 97 hard ticks were collected from *Equidae* (horses and  
135 donkeys). Blood samples were collected in four countries from sub-Saharan Africa (Chad,  
136 Senegal, Djibouti, and Democratic Republic of the Congo [DR Congo] and from France  
137 (Marseille and Corsica) (Figure 1). Hard ticks have only been collected in Chad. All equines  
138 were apparently healthy at the time of sampling (**Table S1**).

139 In Chad, we had two periods of sampling of saddle horses belonging to the Chadian  
140 National Guard (GNT). Ninety-six male horses (from 4 to 15-year-old) were sampled in 2012  
141 and 60 in 2016 (59 male and one female). The infestation of horses by ticks is noticeable  
142 during wet periods especially, with 97 ticks collected in March 2016. In Senegal, an  
143 epidemiological survey, conducted from 2011 to 2014, allowed the inclusion of 126 horses  
144 and 54 donkeys from north-west villages. In Djibouti, in the Decan Reserve, 5 horses and 11  
145 Somali wild donkeys (*Equus asinus somalicus*) were sampled in March 2010. In DR Congo, a  
146 screening survey performed in August 2012 at the Equestrian Club in Kinshasa covered 48  
147 sport and leisure horses (34 males and 14 females from 5 to 33-year-old). In the city of  
148 Marseille (France), a study was conducted in February 2015 on 51 horses (30 males and 21  
149 females from 7 to 35-year-old). Finally, in Corsica (French island in the Mediterranean Sea),  
150 98 horses (59 males and 39 females from 1 to 29-year-old) were sampled in September 2014,  
151 in 13 equestrian centers.

152 The blood sampling was done aseptically using an ad-hoc device (BD Vacutainer®  
153 system; Becton Dickinson, Franklin Lakes, New Jersey, USA) and a single use needle (20G –  
154 0,9X40 mm) at the jugular vein. Vacuum tubes with EDTA K3 were used to collect the blood.  
155 Then, specimens were stored and transported at +4°C. On arrival at the laboratory, specimens  
156 were frozen at -80°C.

157 The 97 ticks were collected manually from Chadian horses in 2016 and stored in 70%  
158 ethanol until they were identified under a binocular microscope. We used the available  
159 taxonomic keys and morphometric table to classify the ticks by family, genus and species  
160 (Walker et al., 2003). Each tick was washed three times in distilled water and stored at -20°C  
161 until DNA extraction.

## 162 **2.2 DNA extraction**

163 EZ1 DNA Kits (Qiagen, Courtaboeuf, France) have been used for the DNA extraction.  
164 The DNA was extracted from 200 µl of blood and from half of a tick. DNA extracts were then  
165 stored at -20°C until PCR analysis.

## 166 **2.3 Piroplasmida specific-PCR tools design**

167 In order to detect piroplasms DNA, we designed primers and probe targeting the most  
168 conserved regions of encoding ribosomal RNA genes (28S, 18S, and 5.8S), as well as primers  
169 targeting mitochondrial gene (*cox1*). House-keeping genes that are typically constitutive  
170 genes, required to maintain basal cell function and that are reliable to study piroplasms  
171 phylogeny (Megan E Schreeg et al., 2016).

172 First, a real time PCR assay for the screening of all piroplasms targeting a conserved  
173 region of the 5.8S gene was developed. Then, for species identification, we designed three  
174 conventional PCR assays targeting 969-bp, 750-bp, and 480-720-bp sequences from the 18S,  
175 28S, and *cox1* genes, respectively. In order to detect an eventual co-infection by the two  
176 equine piroplasmosis agents, PCR assays for specific amplification of *B. caballi* were  
177 developed targeting a 2,930-bp sequence of the 28S gene.

178 All primers and probe for real time PCR and conventional PCR assays were designed  
179 using free web Primer3 software, version 4.0 (<http://frodo.wi.mit.edu/primer3/>); their  
180 sequences were listed in **Table 1**. The specificity and sensitivity of all PCR assays were tested  
181 *in silico* using primer-BLAST (NCBI, USA) and were validated using a panel of DNA

182 extracts from several species of piroplasms, arthropods and bacteria, as well as from human,  
183 donkey, horse, cattle, mouse and dogs (**Table S2**).

#### 184 **2.4 Piroplasmid DNA detection, PCR amplification and phylogenetic analysis**

185 The analytical sensitivity of the 5.8S real time PCR was assessed using 10-fold dilution  
186 from horse infected blood, which harbored 1.64 E+5 parasites per ml (100ul of blood  
187 extracted and eluted in 50ul using EZ1 DNA Kits (Qiagen, Courtaboeuf, France).

188 The initial screening was performed using the 5.8S-based real time PCR assay. Then,  
189 the identification of *Piroplasmida* species in real time PCR-positive samples was based on the  
190 amplification of a 969-bp fragment of 18S rRNA gene. Finally, the 28S rRNA and *cox1* genes  
191 were amplified from selected samples representing different genetic variants based on the 18S  
192 rRNA sequence analysis.

193 Reaction mix for the 5.8S real-time PCR contained 5 µl of the DNA template, 10 µl of  
194 Eurogentec Takyon™ Mix (Eurogentec, Liège, Belgium), 0.5 µl (20 µM) of each reverse and  
195 forward primers, 0.5 µl (5 µM) of the FAM-labeled probe (**Table 1**) and 3.5µl of distilled  
196 water DNase and RNase free, for a final volume of 20 µl. The real time PCR amplification  
197 was carried out in a CFX96 Real-Time system (Bio-Rad Laboratories, Foster City, CA, USA)  
198 using the following thermal profile: Incubation at 50°C for two minutes for UDG action  
199 (eliminating PCR amplicons contaminant), then activation step at 95°C for three minutes,  
200 followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing-extension at 60°C  
201 for 30 seconds.

202 Conventional PCR amplifications were performed in a Peltier PTC-200 model thermal  
203 cycler (MJ Research Inc., Watertown, MA, USA). PCR reactions contained 5 µl of the DNA  
204 template, 25 µl of Amplitaq-Gold STAR™ Mix (Eurogentec, Liège, Belgium), 10 µM (1 µl)  
205 of each primer, and 18 µl of distilled water DNase and RNase free.

206 Conditions for conventional PCR were one incubation step at 95°C for 15 minutes, 40  
207 cycles of one minute at 95°C, 30 seconds annealing at a different hybridization temperature

208 for each PCR assay and one minute at 72°C followed by a final extension for five minutes at  
209 72°C (**Table 1**). Negative and positive controls were included in each molecular assay. The  
210 success of amplification was confirmed by electrophoresis on a 1.5% agarose gel. The  
211 purification of PCR products was performed using NucleoFast 96 PCR plates (Macherey-  
212 Nagel, Hoerd, France) according to the manufacturer's instructions.

213 Co-infections were confirmed by amplifying different species from the same DNA after  
214 cloning using PGEM easy vector system II A1380 (Promega, Charbonnières-les-Bains,  
215 France). The amplicons were sequenced using the Big Dye Terminator Cycle Sequencing Kit  
216 (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an ABI automated sequencer  
217 (Applied Biosystems). The obtained sequences were assembled and edited using ChromasPro  
218 software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia). Then, sequences  
219 were compared with those available in the GenBank database by NCBI BLAST  
220 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Our sequences obtained from positive samples in  
221 addition to sequences of validated piroplasms species already available in Genbank were  
222 aligned using Bioedit software version 7.0.5.3 (ClustalW multiple alignment) (Tom Hall,  
223 2011). For taxonomic analyses, the Maximum-likelihood phylogenetic trees constructed using  
224 MEGA software version 7.0.21(Kumar et al., 2016)with 100 bootstrap replications and  
225 species position confirmed using Topali software version V2.5 (2.5.13.04.03) (Milne et al.,  
226 2009). More specific information found in front of figures.

## 227 **2.5 Statistical analysis**

228 The Epi Info version 7 program (<http://www.cdc.gov/epiinfo/index.html>) was used to  
229 compare prevalence. A difference was statistically significant when p-values were <0.05.

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### 234 3. Results

#### 235 3.1 Sensitivity and specificity of PCR assays

236 The 5.8S real time PCR, as well as the standard 18S RNA-based PCR assay, allowed  
237 the amplification of the following piroplasmids: *T. equi*, *B. caballi*, *Theileria ovis*, *Theileria*  
238 *annulata*, *Theileria buffeli*, *Babesia canis*, and *Babesia vogeli*, all are already identified and  
239 sequenced in our laboratory (**Table S2**). All 18S amplicons from controls were sequenced.  
240 The obtained sequences allowed an accurate identification of all controls. None of the DNA  
241 extracts from negative controls were amplified.

242 The real time PCR is able to detect the presence of parasites with an efficiency of  
243 121.5%, slope: 2.895, Y.int: 41.44 and an almost perfect coefficient higher than 0.98. The  
244 limit of detection was fixed at 1.64E+2 parasites/ml in blood which means 0.82~1 copy per  
245 reaction (**Supp. Table S5**) To insure the reproducibility of the results, samples from the  
246 DR Congo (n=48) were tested simultaneously by 5.8S real time PCR and 18S PCR tools. Both  
247 gave the same result (21 positive on 48 samples). None of them missed positive samples; 5.8S  
248 real time PCR tool was then used for screening in all our samples.

#### 249 3.2 Results of the PCR tools on the samples

250 In total, 548 blood samples of *Equidae* were tested for the presence of piroplasms' DNA  
251 using the real time PCR assay. 29.56% were found positive, including 54 samples from  
252 France and 108 from sub-Saharan Africa. The overall results on blood samples are presented  
253 in **Table 2**.

254 In France, real time PCR screening detected 15/51 (29.4%) positive horses in Marseille,  
255 and 39/98 (39.8%) positive horses from Corsica. There is no statistically significant difference  
256 in the prevalence of piroplasm between these two studied sites (**Table 3**).

257 In sub-Saharan Africa, we found 23/180 (12.8%) positive *Equidae* from Senegal and  
258 21/48 (43.75%) positive horses from DR Congo. Only one horse of 16 (6.25%) *Equidae* from  
259 Djibouti was tested positive. All 11 wild Somali donkeys were found negative. In Chad, the

260 prevalence was significantly higher in 2016 (43/59, 72.9%) than in 2012 (20/96, 20.8%),  
261  $p < 0.0001$  (**Table S3**).

262 Considering the species of *Equidae* we studied, the prevalence of piroplasm was  
263 significantly higher in horses (**160/484**, 33.06%) than in donkeys (**2/64**, 3.12%),  $p \leq 0.0001$ .  
264 The infection was also observed more often in females (**46/124**, 37.09%) than in males  
265 (**116/424**, 27.36%),  $p < 0.20$ . More detailed data are given in **Table 3**.

266 Of the 97 ticks collected in Chad (2016), only 9 (8 female and one male ticks) were  
267 positive (9.27%). Interestingly, all positive ticks were *Rhipicephalus decoloratus*, which  
268 were collected from two horses (coded MGM04 and MGM10) that were also found positive  
269 for piroplasms.

### 270 **3.3 Piroplasms species identification**

271 To identify piroplasms species, all positive samples were subjected to standard PCR  
272 (targeting 18S rRNA gene) coupled with sequencing. Sequencing results showed the  
273 existence of a large genetic diversity (**Table 4**) allowing us to identify four piroplasms  
274 species: *T. equi*, *B. caballi* and two potential new species; provisionally named here *Theileria*  
275 sp. "*Africa*" and *Theileria* sp. "*Europa*". The highest prevalence was observed for *Theileria*  
276 sp. "*Africa*" (51.85%, 84/162) followed by *T. equi* (28.39%, 46/162), *Theileria* sp. "*Europa*"  
277 (19.14%, 31/162), and *B. caballi* (0.62%, 1/162). Only one horse from Senegal was positive  
278 for *B. caballi*.

279 We selected some samples corresponding to each 18S genetic variant to sequence using  
280 28S and *cox1* genes (**Table 1**). All sequences were submitted to the Genbank and their  
281 accession numbers were obtained (**Table S3**). A horse from Senegal was co-infected by *T.*  
282 *equi* and *B. caballi* which is confirmed by amplifying the two species from the same DNA  
283 after cloning.

284 **3.4 Phylogenetical analysis**

285 The phylogenetic trees constructed on the base of 18S, 28S and *cox1* genes allowed the  
286 position of the identified species to be visualized (Figures 2, 3, and 4, respectively). Both  
287 *Theileria* sp. "Africa" and *Theileria* sp. "Europa" formed separate clades with samples of  
288 different geographical origins grouped together with a good bootstrap support (Figures 2, 3,  
289 and 4).

290 In a second time, we compared sequences of three genes of *Theileria* spp. identified in  
291 the present study with *T. equi* sequences of WA strain and USDA strains available in the  
292 Genbank (**Table S4**). We discovered that the differences among *T. equi* and the two potential  
293 new species varied between 4 to 14%.

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#### 310 4. Discussion

311 To the best of our knowledge, all published PCR-based assays are able to amplify either  
312 only one species or a group of piroplasms (Quorollo et al., 2017; Salem et al., 1999). Herein, we  
313 have developed a real time PCR assay targeting the 5.8S gene able to detect well-known  
314 equine pathogens (*T. equi* and *B. caballi*), well-known mammal pathogens as well as potential  
315 new species. The 5.8S gene is a conservative gene easy to amplify by PCR using one pair of  
316 universal primers (Gou et al., 2012). For the design of our 5.8S real time PCR assay, highly  
317 divergent regions flanked by highly conserved regions among piroplasms have been targeted  
318 from 32 aligned sequences of *Theileria* spp. and *Babesia* spp. The designed real time PCR  
319 assay exhibited a good sensitivity. As expected *in silico*, the tool allowed an accurate *in vitro*  
320 detection of all known piroplasms, as well as those not yet described. Besides, none of the  
321 DNA extracts from the control group were amplified with this molecular tool confirming its  
322 specificity. The *cox1* gene is one of the most used markers for population genetics and  
323 phylogeographic studies (Derycke et al., 2010). The 18S and 28S genes, generally considered  
324 to be highly conserved, are actually composed of a mixture of conserved and divergent  
325 regions (Gou et al., 2012). For the design of the 28S and *cox1* PCR assays, 35 and 34  
326 sequences were respectively aligned. The molecular phylogeny of *Babesia* and *Theileria* is  
327 usually based on 18S sequences (Gou et al., 2012). For the design of our 18S PCR assay, the  
328 combination of primers F1 and R4 showed high specificity.

329 Overall, we analyzed 548 blood specimens from apparently healthy *Equidae* sampling  
330 in four sub-Saharan African countries (DR Congo, Djibouti, Chad, and Senegal) and France  
331 (Marseille and Corsica). The overall prevalence of piroplasms detected in blood samples was  
332 29.6% (162/548). Only one case of *B. caballi* was diagnosed in Senegal, in a horse. All the  
333 other samples were positive for *Theileria* spp. Thus, the overall prevalence of *Theileria* spp.  
334 was 28.4%. Besides, two new potential *Theileria* species were detected, *Theileria* sp.  
335 "*Europa*" and *Theileria* sp. "*Africa*". *Theileria* sp. "*Europa*" exhibited a prevalence of 5.7%

336 (31/548). *Theileria* sp."Africa" was more commonly observed with a prevalence of 15.3%  
337 (84/548). This higher prevalence observed in Chadian horses in 2016 compared to 2012 can  
338 be explained by the inclusion of another sampling site such as Amguifel (**Table S1**). Previous  
339 studies reported PCR-negative samples while indirect fluorescent antibody (IFA) tests were  
340 positive. One of the hypotheses was the existence of variants that have not yet been identified  
341 and therefore cannot be detected by the available PCR tools (Bhoora et al., 2010). Indeed, we  
342 have identified here different genotypes that can easily be omitted by conventional PCR tools  
343 (**Table 4**).

344 Ticks from the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus* are natural vectors  
345 of *Babesia* and *Theileria* (Tamzali, 2013). The 97 ticks studied from Chad belonged to the  
346 two following genera: *Hyalomma* (4.1%, 4/97) and *Rhipicephalus* (95.9%, 93/97). Nine ticks  
347 (9.3%, 9/97) identified as *Rh. decoloratus* were found positive for *Theileria* sp. "Africa".  
348 Besides, these ticks were found on two horses which were also positive for *Theileria* sp.  
349 "Africa". More studies on these ticks must confirm or disprove their capacity to transmit  
350 piroplasms.

351 Our study does not claim to be representative because of the reduced number of  
352 *Equidae* tested. Furthermore, our sampling was carried out in an opportunistic manner but it  
353 gives a picture on the epidemiological situation in the studied areas. The horses tested are  
354 certainly protected by a pre-immunization while latent infection persists. Tick infestations are  
355 regularly observed in areas where our study has been conducted and sporadic clinical cases of  
356 piroplasmosis are reported by veterinarians. It is therefore important to limit the infestation by  
357 ticks with external deworming measures to control the diseases they transmit. Asymptomatic  
358 infection is very persistent (this is known for *T. equi*) and tick infestation (especially nymphs)  
359 may pass unnoticed.

360 The sole case of *B. caballi* was identified in a horse from Senegal, which was co-  
361 infected with *T. equi*. We should notify that the sampling time plays a critical role in the  
362 detection of circulating parasites. Indeed, infections by *B. caballi* are self-limiting. Horses are  
363 generally able to eliminate the infection within one to three years naturally or after a  
364 sterilizing treatment (Friedhoff and Soulé, 1996).

365 *T. equi* and *B. caballi* are the parasites most frequently involved in equine  
366 piroplasmosis. However, recent studies have reported the existence of other piroplasms  
367 species in horses. Indeed, *B. canis* has recently been reported in horses in France and Italy and  
368 *B. capreoli* in Italy (Fritz, 2010; Zanet et al., 2017). The analysis of three different genes of  
369 the two-potential new *Theileria* genotypes clearly showed a distant position between these  
370 species and the others. These data strongly support the fact that they differ from other  
371 recognized species, including *T. equi* (**Table S4**). New species are isolated from equines and  
372 described and validated using phylogenetic analyses based on both 18S rDNA sequences and  
373 223 nuclear-encoded protein-coding genes, extensive genome-wide differences and with  
374 predicted protein divergence with *T. equi* (Knowles et al., 2018). Finally, the pathogenicity of  
375 these potentially new species remains unknown.

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385 **5. Conclusion**

386 Overall, sensitive and specific PCR assays have been developed to potentially identify  
387 all piroplasm species and to study their phylogeny. Four horse-infecting piroplasms, including  
388 two potentially new species, were identified in *Equidae* from sub-Saharan Africa and France.  
389 However, few epidemiological investigations on equine piroplasmosis are conducted. Our  
390 study provides a better understanding of the situation. Further studies, covering larger  
391 geographical areas and larger number of samples, are nevertheless required to improve  
392 exploration of the health status of *Equidae*. These studies will assess more specifically the  
393 actual prevalence of equine piroplasmosis and its impacts on equine populations. Moreover,  
394 the establishment of high rigorous prophylaxis plans, allowing both surveillance and control  
395 of equine piroplasmosis should be proposed. The involvement of public authorities in the  
396 detection of these diseases remains essential to contain them, or at least to reduce their spread.

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517

518 **Figure legends.**

519 **Figure 1.** Map of countries where samples were collected in sub-Saharan Africa and Europe.

520 **Figure 2.** Maximum-likelihood phylogenetic tree of piroplasms, including our new species  
521 based on partial 858-bp 18S gene.

522 **Figure 3.** Maximum-likelihood phylogenetic tree of piroplasms, including our new species  
523 based on partial 754-bp 28S gene.

524 **Figure 4.** Maximum-likelihood phylogenetic tree of piroplasms, including species identified  
525 in the present study based on partial 480-bp *cox1* gene.

Fig 1

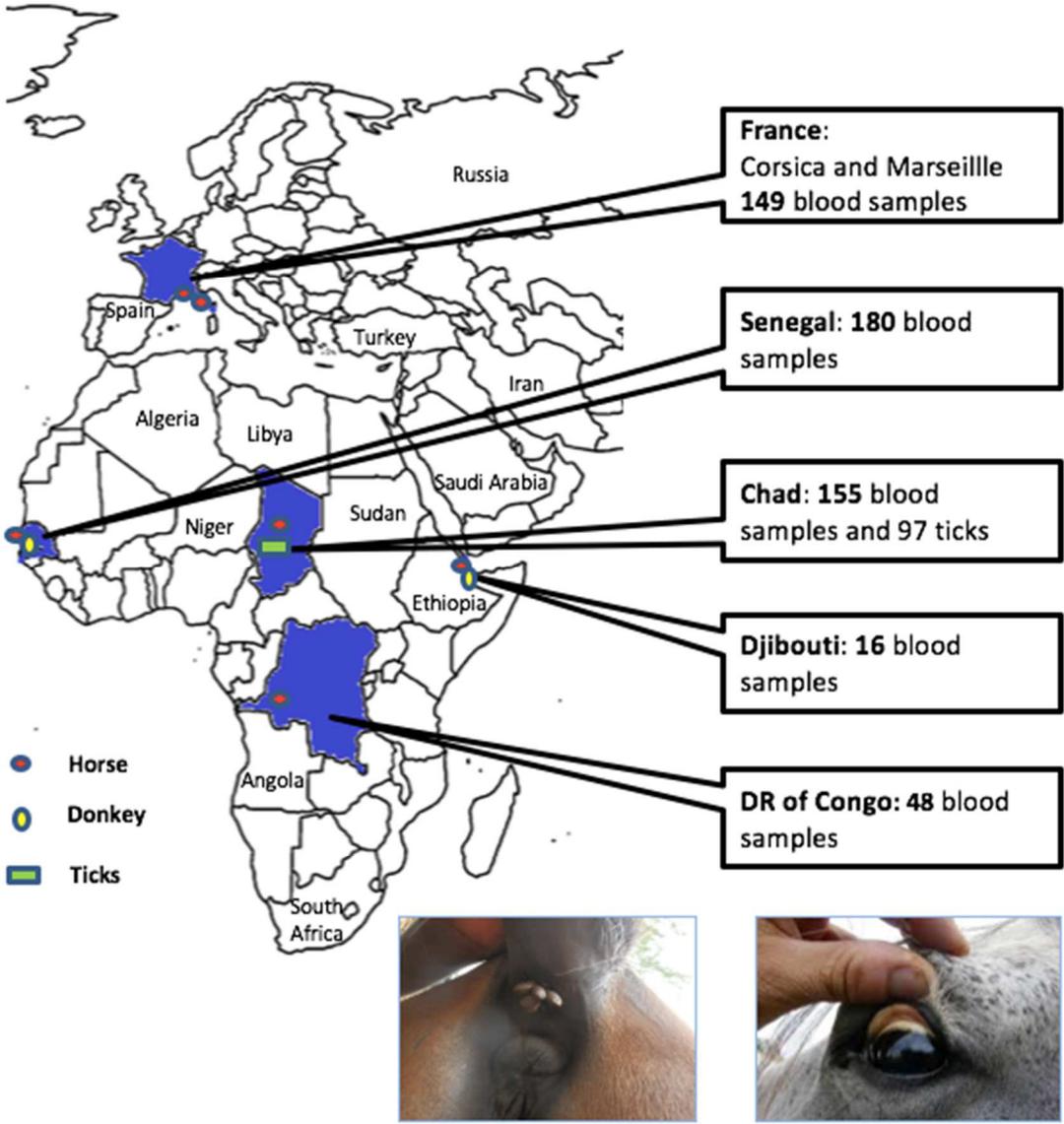
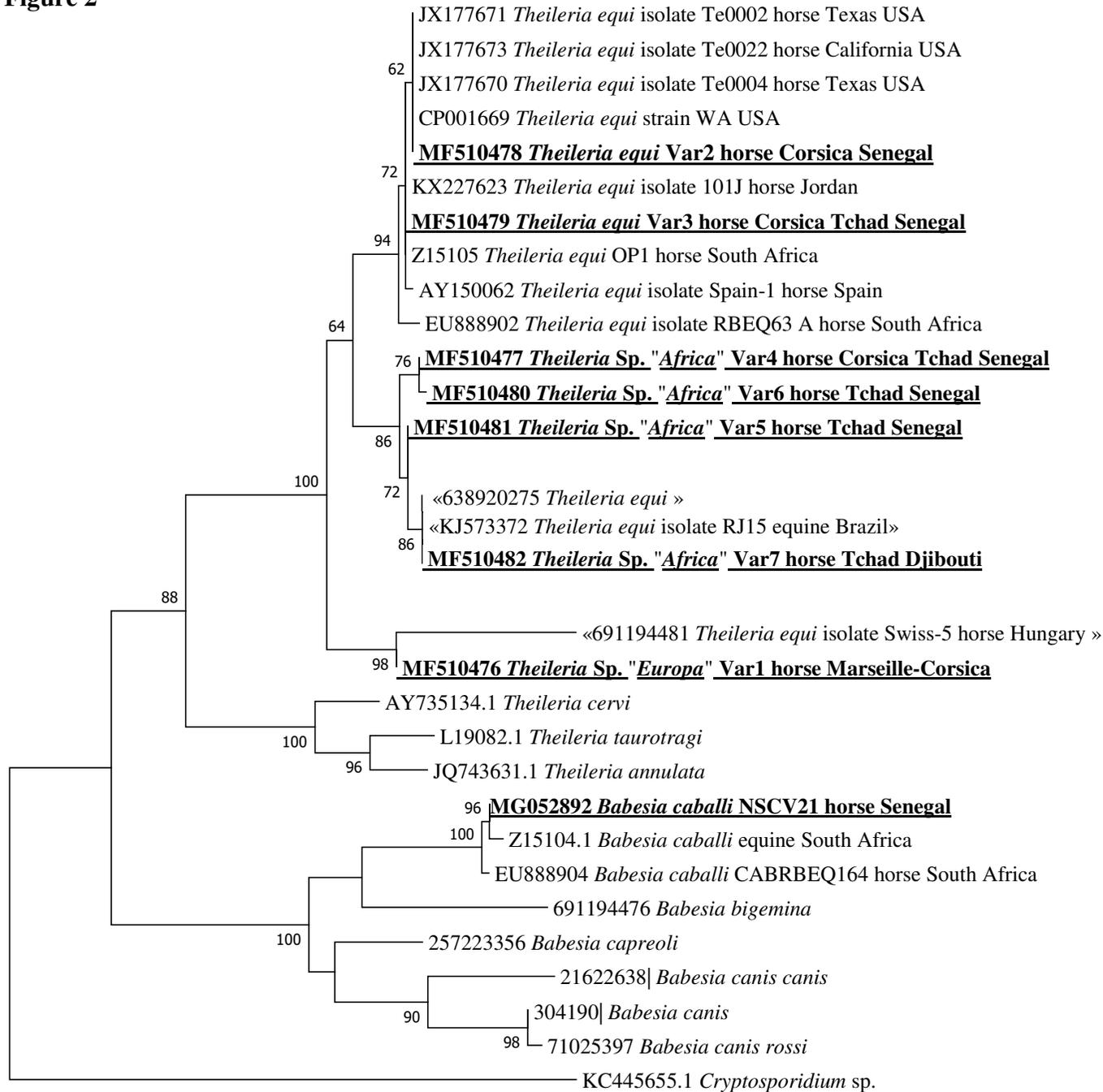


Figure 2



**Figure 3**

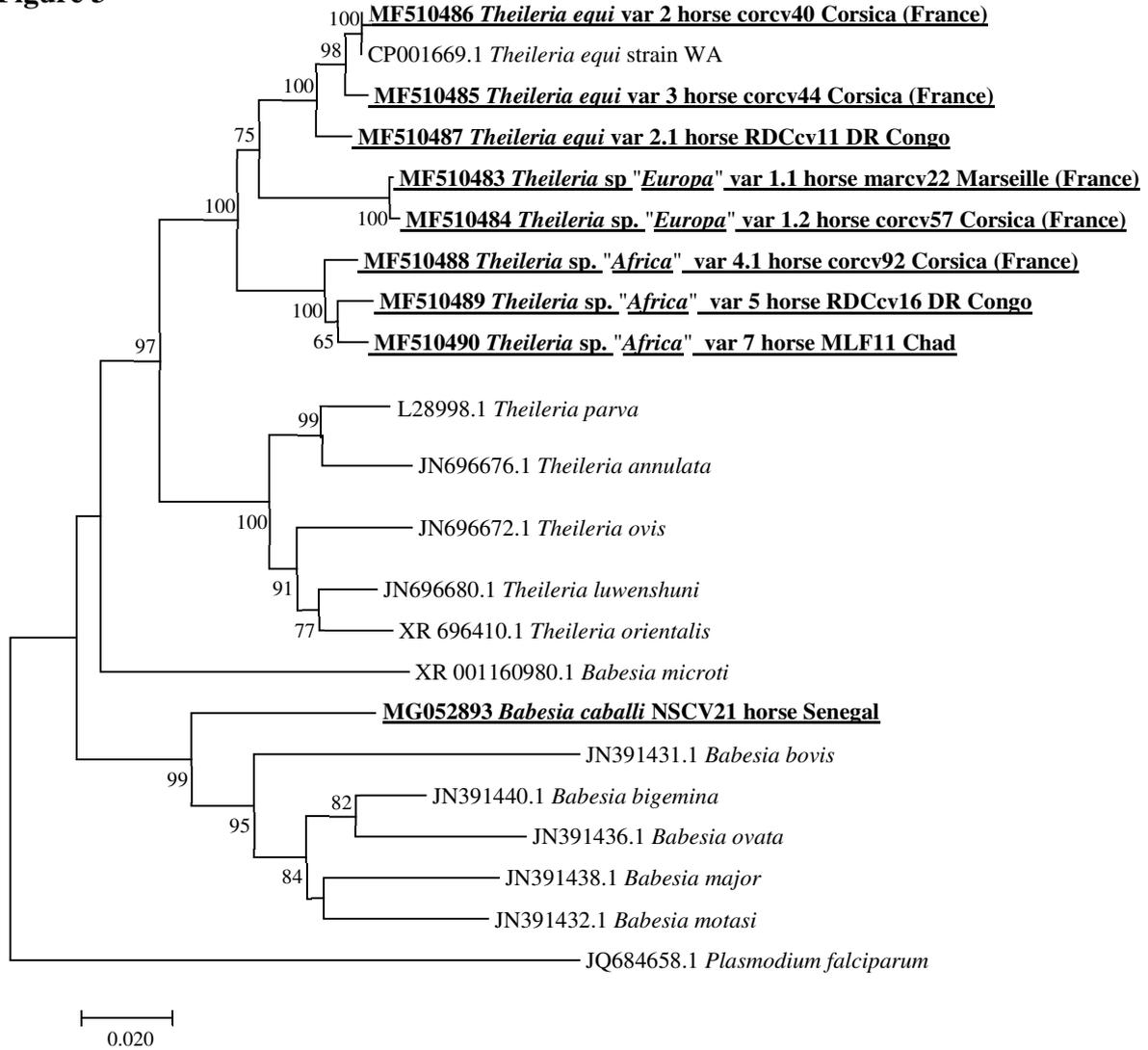
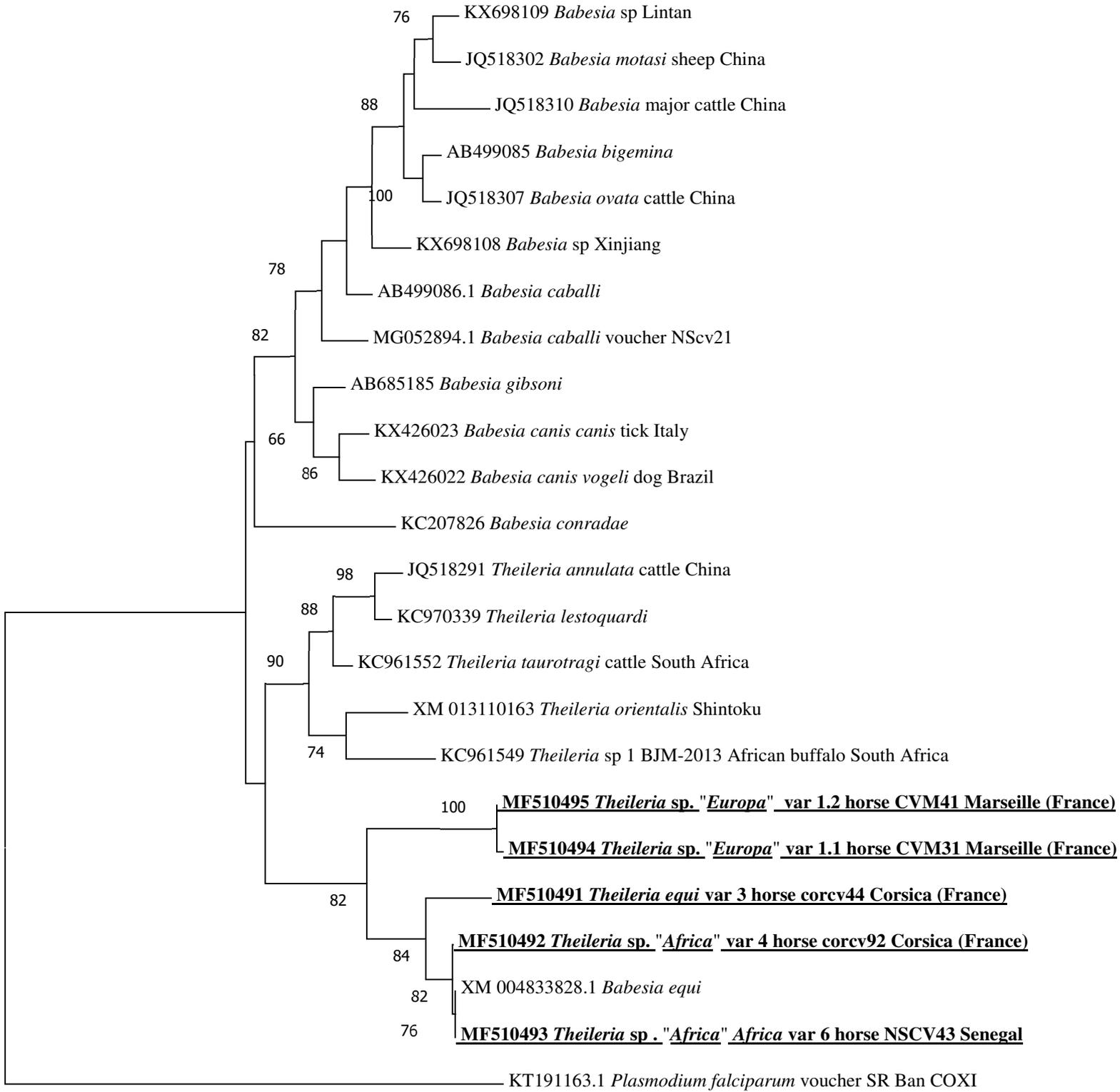


Fig 04



0.10

**Table 1.** Oligonucleotide sequences of primers and probe used for qPCRs and conventional PCRs in this study.

Targets	Targeted gene	Name	Primers (5'-3') and probe	Annealing temperature
<i>Piroplasmida</i>	5.8S	5.8S-F5	AYYKTYAGCGRTGGATGTC	60°C
		5.8S-R	TCGCAGRAGTCTKCAAGTC	
		5.8S-S	FAM-TTYGCTGCGTCCTTCATCGTTGT-MGB	
	18S	piro18S-F1	GCGAATGGCTCATTAIACA	58°C
		piro18S-F4	CACATCTAAGGAAGGCAGCA	
		piro18S-F3	GTAGGGTATTGGCCTACCG*	
		piro18S-R3	AGGACTACGACGGTATCTGA*	
	28S	28S-F2	CACCCCYGKCCGTACDGAT	58°C
		28S-R2	CCTCAYTGAGYTYGCCTTRGGAC	
<i>B. caballi</i>	28S	28Scab-F4	GCAGAAAAGAAAATAACCATG	62°C
		28Scab-R3	GCGTTCAGTCATTATCCAACGG	
		28Scab-F6	GCTTTAGCACGGTTGCTAGGA*	
		28Scab-F7	GGCATAGCAGTCCGGYYTTCGG*	
		28Scab-F8	TGTCCCTATCTGCCATCTAG*	
		28Scab-R6	TCGCCCCCTATACCCGGATTTG*	
		28Scab-R7	CCGAARRCCGGACTGCTATGCC*	
	<i>cox1</i>	piro-F	AGGAAGTGGWACWGGITGGA	57°C
		piro-R	GATGISCCCAIACIARACAWCC	
<i>Theileria</i> sp. "Europe"	<i>cox1</i>	383-F3	GAAGTGGWACHGGDTGGAC	57°C
		900-R1	CCHGADGTATACATATGRTG	
<i>Theileria</i> sp. "Africa" & <i>Theileria equi</i>	<i>cox1</i>	COI-F	GTGAYGTTGTTTTTCCAAG	57°C
		COI-R	CCWGTGTGTACCTCCAAYDAC	

\*Primers used for sequencing only

**Table 2.** Overall prevalence of piroplasms in blood and tick samples.

<b>Country</b>	<b>Blood</b>	<b>Ticks</b>
	<b>Number of positive / Number of tested samples (%)</b>	
<b>France (Marseille)</b>	15/51 (29.4)	NA
<b>France (Corsica)</b>	39/98 (39.8)	NA
<b>Senegal</b>	23/180 (12.8)	NA
<b>DR Congo</b>	21/48 (43.75)	NA
<b>Chad (2012)</b>	20/96 (20.8)	NA
<b>Chad (2016)</b>	43/59 (72.9)	9/97 (9.27)
<b>Djibouti</b>	1/16 (6.25)	NA
<b>Total</b>	162/548 (29.6)	9/97 (9.27)

NA: Not available

**Table 3.** Prevalence of piroplamosis agents according to the country, animals and their sex.

	<b>Horses</b>	<b>Donkeys</b>	<b>X<sup>2</sup> test</b>
	Number of positive / Number of tested samples (%)		
<b>Senegal</b>	22/127 (17.32%)	1/53 (1.87%)	P ≤ 0,02
<b>DR Congo</b>	21/48 (43.75%)	0	Only horse
<b>Marseille (France)</b>	15/51 (29.41%)	0	Only horse
<b>Corsica (France)</b>	39/98 (39.8%)	0	Only horse
<b>Chad in 2012</b>	20/96 (20.83%)	0	Only horse
<b>Chad in 2016</b>	43/59 (72.88%)	0	Only horse
<b>Djibouti</b>	0/5 (0%)	1/11 (9.1%)	P ≤ 0,90
	<b>Male</b>	<b>Female</b>	<b>X<sup>2</sup> test</b>
	Number of positive / Number of tested samples (%)		
<b>Senegal</b>	13/127 (10.57%)	10/53 (18.87%)	p ≤ 0.20
<b>DR Congo</b>	13/34 (38.24%)	8/14 (57.14%)	p ≤ 0.5
<b>Marseille (France)</b>	8/33 (24.24%)	7/18 (38.89%)	p ≤ 0.5
<b>Corsica (France)</b>	18/59 (30.5%)	21/39 (53.85%)	p ≤ 0.20
<b>Chad in 2012</b>	20/96 (20.83%)	0	Only male
<b>Chad in 2016</b>	43/59 (72.88%)	0	Only male
<b>Djibouti</b>	1/16 (6.25%)	0	Only male

**Table 4.** Piropasms genetic variants infecting equines found in studied areas.

Genetic variants	Areas						Total
	Senegal	DR Congo	Chad	Djibouti	Corsica	Marseille	
	Number of positive in this variant / Total of positives (%)						
	13/23 (56.5)	3/21 (14.28)	5/63 (7.9)	0/1 (0)	11/39 (28.2)	0/15 (0)	32/162 (19.8)
<i>Theileria equi</i> (var3)	1/23 (4.3)	0/21 (0)	7/63 (11.1)	0/1 (0)	7/39 (17.9)	0/15 (0)	15/162 (9.3)
<i>Theileria</i> sp. "Africa" (var4)	1/23 (4.3)	0/21 (0)	14/63 (22.2)	0/1 (0)	6/39 (15.3)	0/15 (0)	21/162 (12.9)
<i>Theileria</i> sp. "Africa" (var5)	3/23 (1,3)	17/21 (80.9)	5/63 (7.9)	1/1 (100)	0/39 (0)	0/15 (0)	26/162 (16)
<i>Theileria</i> sp. "Africa" (var6)	4/23 (17,4)	1/21 (4.76)	22/63 (34.9)	0/1 (0)	0/39 (0)	0/15 (0)	27/162 (16.6)
<i>Theileria</i> sp. "Africa" (var7)	0/23 (0)	0/21 (0)	10/63 (15.8)	0/1 (0)	0/39 (0)	0/15 (0)	10/162 (6.2)
<i>Theileria</i> sp. "Europa" (var1)	0/23 (0)	0/21 (0)	0/63 (0)	0/1 (0)	14/39 (35.9)	15/15 (100)	29/162 (17.9)
<i>Theileria</i> sp. "Europa" (var1.2)	0/23 (0)	0/21 (0)	0/63 (0)	0/1 (0)	1/39 (2.6)	0/15 (0)	1/162 (0.6)
<i>Babesia caballi</i>	1/23 (4.3)	0/21 (0)	0/63 (0)	0/1 (0)	0/39 (0)	0/15 (0)	1/162 (0.6)
<b>Total</b>	23	21	63	1	39	15	<b>162</b>

**Table 5.** Prevalence of piroplasms in few European and African countries.

Country	Year	Techniques	Samples	<i>T. equi</i>	<i>B. caballi</i>	References
<b>South Africa</b>	2010	Nested PCR	41 horses	80%	72%	(Bhoora et al., 2010)
<b>Sudan</b>	2013	Conventional PCR (18S)	499 <i>Equidae</i>	35.95%	0%	(Salim et al., 2013)
<b>Egypt</b>	2016	Nested PCR	88 horses 51 donkeys	36.4% 43.1%	19.3% 15.7%	(Mahmoud et al., 2016)
<b>Kenya</b>	2015	Nested PCR (18S)	71 donkeys 16 zebras	72% 100%	0% 0%	(Hawkins et al., 2015)
<b>Nigeria</b>	2014	Giemsa blood smear	240 horses	40%	2.5%	(Turaki et al., 2014)
<b>Tunisia</b>	2013	Reverse Line Blot hybridization	104 horses	11.53	1.92	(Ros-García et al., 2013)
<b>Italy</b>	2016	Real time PCR	673 horses	27.49%	4%	(Bartolomé Del Pino et al., 2016)
<b>Italy</b>	2017	Semi-nested PCR	135 horses	13.33%	0%	(Zanet et al., 2017)
<b>Turkey</b>	2017	Multiplex PCR	125 horses	8.8%	0%	(Güven et al., 2017)
<b>Portugal</b>	2013	ELISA	162 horses	17.9%	11.1	(Ribeiro et al., 2013)
<b>France</b>	2010	Conventional PCR (18S)	166 dogs	19%	0.6%	(Fritz, 2010)
	2010	Conventional PCR (18S)	111 horses	80%	1.2%	(Fritz, 2010)
<b>Spain</b>	2017	Indirect Fluorescent Antibody Test	3,100 horses	44%	21%	(Guadalupe et al., 2017)