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1 **Co-infection of bacteria and protozoan parasites in *Ixodes ricinus* nymphs collected in**  
2 **the Alsace region, France**

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24 **Keywords:** *Ixodes ricinus*; Co-infection; Bacterial pathogens; Protozoan parasites; MALDI-  
25 TOF MS.

26 **Abstract**

27 Fifty nymphal *Ixodes ricinus* ticks collected in Alsace, France, identified by morphological  
28 criteria and using MALDI-TOF MS, were tested by PCR to detect tick-associated bacteria and  
29 protozoan parasites. Seventy percent (35/50) of ticks contained at least one microorganism;  
30 26% (9/35) contained two or more species. Several human pathogens were identified  
31 including *Borrelia burgdorferi* s.s. (4%), *Borrelia afzelii* (2%), *Borrelia garinii* (2%),  
32 *Borrelia valaisiana* (4%), *Borrelia miyamotoi* (2%), *Rickettsia helvetica* (6%) and “*Babesia*  
33 *venatorum*” (2%). *Bartonella* spp. (10%) and a *Wolbachia* spp. (8%) were also detected. The  
34 most common co-infections involved *Anaplasmataceae* with *Borrelia* spp. (4%),  
35 *Anaplasmataceae* with *Bartonella* spp. (6%) and *Anaplasmataceae* with *Rickettsia* spp. (6%).  
36 Co-infection involving three different groups of bacteria was seen between bacteria of the  
37 family *Anaplasmataceae*, *Borrelia* spp. and *Bartonella* spp. (2%). Results highlight the panel  
38 of infectious agents carried by *Ixodes ricinus*. Co-infection suggests the possibility of  
39 transmission of more than one pathogen to human and animals during tick blood feeding.

40

## 41 **Introduction**

42 *Ixodes ricinus*, the European sheep tick, is widely found in temperate regions of the northern  
43 hemisphere. It has a high attraction to biting humans (Parola and Raoult, 2001) and well  
44 known in Europe as the vector of *Borrelia burgdorferi* sensu lato (s.l.), the etiologic agent of  
45 Lyme disease (Stanek et al., 2012), tick-borne encephalitis (Amicizia et al., 2013) and  
46 granulocytic anaplasmosis (Jin et al., 2012). The Alsace and Meuse areas, in north-eastern  
47 France, are the most endemic regions of the country with more than 100 cases per 100,000  
48 inhabitants between 2009 and 2011 (HCSP, 2014). Moreover, *I. ricinus* ticks have been  
49 reported to harbour or transmit other emerging human pathogens such as *Rickettsia helvetica*,  
50 *Rickettsia monacensis* and *Babesia divergens* (Rizzoli et al., 2014). The emergence of tick-  
51 borne diseases in the past decade is worrying (Silaghi et al., 2016) and has underlined the  
52 need to strengthen epidemiological and vector surveys. A recent study conducted in the region  
53 of the French Ardennes on *I. ricinus* revealed high microorganism co-infection rates in adult  
54 ticks, raising questions about the possible co-transmission of these agents to humans or  
55 animals, and the potential consequences for human and animal health (Moutailler et al.,  
56 2016). The aim of the present work was to test nymphal *I. ricinus* stage infections by *Borrelia*  
57 spp. and other associated microorganisms circulating in an area of the Alsace region using  
58 molecular tools.

59

## 60 **Materials and methods**

61 Ticks were collected using the drag flag technique (Parola and Raoult, 2001) between May  
62 and June 2014 in the region of Murbach: N 47.918961 ; E7.210436 ; Altitude 520 m (Haut-  
63 Rhin, Alsace, France) in the frame of the study of Goldstein et al. 2018 (Goldstein et al.,  
64 2018). A convenient sample of 50 nymphs were selected for the current study, to launch a  
65 collaboration between two reference centers in France. Each specimen was morphologically

66 identified using standard taxonomic keys (Hylliard,1996). Proteomic identification using  
67 MALDI-TOF MS (Matrix-assisted laser desorption/ionization mass spectrometry) was  
68 conducted as previously described (Yssouf et al., 2013). DNA from these specimens was then  
69 extracted using the EZ1<sup>®</sup> Tissue Kit and BioRobot<sup>®</sup> EZ1<sup>®</sup>(QIAGEN, Hilden, Germany)  
70 according to the manufacturer's instructions. To assess the presence of the DNA of common  
71 microorganisms in ticks, all extracted DNA were screened by real-time polymerase chain  
72 reaction (qPCR), targeting specific genes of *Borrelia* spp. (*16S rRNA*), *Rickettsia* spp. (*gltA*),  
73 *R. helvetica* (*rfbE*), *Bartonella* spp. (*ITS*), *Bartonella henselae* (*Pap31*), Anaplasmataceae  
74 (*23S*), *Anaplasma phagocytophilum* (*polA*), *Coxiella burnetii* (*IS30A*) and *Babesia* spp.  
75 (*18SrDNA*), as previously described (Aubry et al., 2016). Negative controls (qPCR mix and  
76 DNA of uninfected *Rhipicephalus sanguineus* from laboratory colony) and positive controls  
77 (*Borrelia crocidurae*, *Bartonella elizabethae*, *R.monacensis*, *A. phagocytophilum*, *Babesia*  
78 *microti* and *C. burnetii* DNA originating from laboratory collection) were used in each  
79 respective qPCR. Ticks analyzed by qPCR were considered to be positive when the cycle  
80 threshold (Ct) value was less than or equal to 36, as used in previous studies (Aubry et al.,  
81 2016). All ticks which were identified as being positive by qPCR were confirmed by  
82 conventional PCR amplifications and sequencing. Sequences were analyzed as described  
83 previously (Aubry et al., 2016).

84

## 85 **Results**

86 All specimens were morphologically identified as the nymphal stage of *I. ricinus*. The  
87 protein spectra profiles from the MALDI-TOF MS analysis of tick legs were consistent and  
88 reproducible with high-intensity peaks in the range of 2–20 kDa (Additional file1). Of them,  
89 34 tick specimens obtained log-score values (LSVs) higher than 1.8 when they were tested  
90 against the home-made reference MS spectra database. These LSVs (>1.8) considered as

91 correctly-identified specimens, corresponded to ticks from *I. ricinus*. For the remaining 16  
92 ticks, the intensity and diversity of MS spectra were insufficient for relevant analysis.

93 Nearly 70% (35/50) of the *I. ricinus* collected were infected by at least one of the  
94 microorganisms tested by the qPCR (Additional file2). A total of 27/50 (54%) specimens  
95 harboured bacteria from the *Anaplasmataceae* family. Positive samples were successfully  
96 amplified through the standard PCR using primers targeting 23S rRNA. However, after the  
97 sequencing, only four high-quality sequences were obtained. The sequences of the remaining  
98 23 amplicons, were of poor quality. Blast analyses of the four high-quality sequences show  
99 99.6% identity with “*Wolbachia* endosymbiont of *Drosophila incompta*” (GenBank accession  
100 no. CP011148). *Borrelia* spp. DNA was found in 20% (10/50) of the ticks. Sequencing  
101 analyses targeting the 16S rRNA gene long fragment led to the identification of seven  
102 sequences including two *B. burgdorferi* s.s. (GenBank accession no. CP019767 with 99.32  
103 and 100% identity), two *B. valaisiana* (GenBank accession no. NR\_036807, with 100%  
104 identity), one *B. afzelii* (GenBank accession no. KX688604, with 100% identity), one *B.*  
105 *garinii* (GenBank accession no. DQ147793, with 99.74% identity) and one *B. miyamotoi*  
106 (GenBank accession no. JF951382, 100% identity). The three remaining sequences of *Borrelia*  
107 spp. were of poor quality and were removed from analyses. Three ticks were found to be  
108 positive for *Rickettsia* spp. *R. helvetica* was found in these three ticks using the *R. helvetica*-  
109 specific qPCR targeting the *rflE* gene.

110 *Bartonella* spp. screening targeting the ITS gene found five positives. These five positive  
111 samples were only successfully amplified using primers targeting the long fragment of the  
112 *ftsZ* gene. None of the *rpoB*, *gltA* or ITS genes were amplified. The *ftsZ* sequences obtained  
113 from these five positive samples had less than 1% mismatches. Blast analyses showed 91.9%  
114 identity with uncharacterized *Bartonella* sp. strain B44608 (KY679153) and *B. vinsonii*  
115 subsp. *vinsonii* (AF467757) and 91.3% identity with *B. vinsonii* subsp. *berkhoffii*

116 (KU292573). Phylogenetic analyses showed that this potentially new *Bartonella* sp. forms a  
117 separate clade between *Bartonella ancashensis* and the other *Bartonellaceae* species despite  
118 belonging to the latter (Figure 1).

119 Lastly, from one tick the DNA of *Babesia* spp. was amplified using primers and probes  
120 targeting the 18S rDNA specific. A fragment of the *18S rRNA* gene was then amplified using  
121 conventional PCR primers. The resulting sequence was blasted and presented 100% identity  
122 with *Babesia* sp. “venatorum” (KU204792) and 98% with *B. odocoilei* (KC460321) detected  
123 in elk blood in Canada (Figure 2). None of the tested ticks was positive for *A.*  
124 *phagocytophilum*, *B. henselae* or *C. burnetii*.

125 The overall prevalence of the pathogens found in this study was as follows: *B. burgdorferi* s.s.  
126 (4%), *B. afzelii* (2%), *B. garinii* (2%), *B. valaisiana* (4%), *B. miyamotoi* (2%), *R. helvetica*  
127 (6%), “*B. venatorum*” (2%), *Bartonella* spp. (10%) and *Wolbachia* spp. (8%).

128 Co-infection between two or three microorganisms was observed among the positive ticks.  
129 Indeed, co-infection by two microorganisms was found in 23% (8/35) of positive ticks. These  
130 include *Borrelia* spp. with *Anaplasmataceae* (2/35) and *Bartonella* spp. with  
131 *Anaplasmataceae* (3/35), followed by *Rickettsia* spp. with *Anaplasmataceae* (3/35). Co-  
132 infection with three microorganisms was found in 3% (1/35) of positive ticks. This multiple  
133 co-infection was found between *Borrelia* spp., *Bartonella* spp. and *Anaplasmataceae*  
134 (1/35)(Additional file2).

135

## 136 **Discussion**

137 Results reported in the present study indicate that *I. ricinus* ticks collected in Alsace, France  
138 were infected with multi-organism bacteria and protozoa (70%). However, the limited number  
139 of tested nymphs cannot reflect a complete epidemiological picture of all bacteria and

140 protozoa circulating in or between *I. ricinus* ticks in this region. Thus, the full spectrum of  
141 bacteria infecting the collected *I. ricinus* has not been identified. That said, the results  
142 reported here present additional knowledge about *I. ricinus* associated microorganisms  
143 circulating in the north-eastern region of France.

144 The high rate of infection (70%) was previously reported in this same region (Ferquel et al.,  
145 2006) and in another neighbouring region (Moutailler et al., 2016). This study confirmed the  
146 presence of a broad spectrum of *Borrelia* species belonging to the Lyme disease group,  
147 namely: *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. valaisiana*, already reported in this  
148 region (Ferquel et al., 2006; Moutailler et al., 2016). We also detected emerging pathogens  
149 such as *B. miyamotoi* and *R. helvetica* as well as a probable symbiotic bacteria belonging to  
150 the *Wolbachia* genus that has 99.6% identity with “*Wolbachia* endosymbiont of *Drosophila*  
151 *incompta*”. Protozoan parasites belonging to the *Babesia* genus were detected in 2% of ticks.  
152 However, phylogenetic analysis of the *18S rRNA* gene revealed that the *Babesia* detected here  
153 is part of a group of “*B. venatorum*” strains that form a separate clade. These strains have  
154 been detected in China (Jiang et al., 2015) and other European countries, such as Italy  
155 (Cassini et al., 2010), Slovenia (Duh et al., 2005), the Netherlands (Kik et al., 2011) and the  
156 Czech Republic (Venclikova et al., 2015), being isolated from *I. ricinus* ticks, roe deer or  
157 both. *Babesia* “*venatorum*”, previously called *Babesia* sp. EU1, is an emerging human and  
158 animal pathogen causing clinical features similar to those of *B. microti* in humans but its  
159 typical clinical course is unknown (Jiang et al., 2015). In France, it was isolated by culture  
160 from infected roe deer in the region of Deux Sèvres (Bonnet et al., 2007) and detected in *I.*  
161 *ricinus* ticks collected in the Île-de-France region (Paul et al., 2016) and in Brittany with a  
162 low prevalence of 0.65% and 0.35% in 2012 and 2013, respectively (Jouglin et al., 2017). A  
163 study conducted in a neighbouring region did not detect “*B. venatorum*” in *I. ricinus* ticks  
164 (Moutailler et al., 2016), suggesting a low prevalence or a recent introduction to the area.

165 *Bartonella* spp. was found in 10% of collected ticks. Only five positive samples in the qPCR  
166 were amplified using the primers targeting the *ftsZ* gene. The phylogenetic tree showed that  
167 this unique sequence of *Bartonella* sp. forms a separate clade between *B. ancashensis* and  
168 other *Bartonellaceae* species. Unfortunately, we did not successfully amplify this *Bartonella*  
169 sp. using other genes (La Scola et al., 2003). The lack of this additional data precludes the  
170 complete description of this potentially new *Bartonella* species. *Bartonella* associated with *I.*  
171 *ricinus* was previously reported (Chomel & Kasten 2010). DNA of *B. henselae*, the agent of  
172 cat scratch disease was detected in 17.6% of ticks in the French Ardennes (Moutailler et al.,  
173 2016) and in 9.8% of ticks collected in northern France (Halos et al., 2005). Interestingly, in  
174 France, *B. henselae*, *B. doshiae*, *B. tribocorum* and *B. schoenbuchensis* were isolated from  
175 blood samples of patients with poorly qualified syndromes after tick bites (Vayssier-Taussat  
176 et al., 2016).

177 Co-infections with two or three microorganisms were identified in 18% of *I. ricinus*  
178 nymphs (9/50), which accounted for 26% (9/35) of all infected nymphs. This co-infection  
179 could be the result of co-feeding or feeding on an infected host by several pathogens  
180 (Raileanu et al., 2017). *Anaplasmataceae* were involved in almost all combinations of co-  
181 infections. The association of three bacteria was seen between *Anaplasmataceae*, *Borrelia*  
182 spp. and *Bartonella* spp. (2%). In France, *B. burgdorferi* s.l. and *Bartonella* spp. have  
183 previously been simultaneously detected in 1.3% of nymphs collected in Lille (Halos et al.,  
184 2005). In other European countries, the prevalence of co-infection with different bacteria in *I.*  
185 *ricinus* nymphs has been reported. Indeed, the rate reported in Romania was 17.1% (Raileanu  
186 et al., 2017); in Italy, the prevalence was 5.71% (Otranto et al., 2014), whereas in western  
187 Switzerland 4.6% of the nymphal *I. ricinus* collected were found to be infected with several  
188 microorganisms (Lommano et al., 2012). In Spain, one nymph was co-infected with *B.*  
189 *valaisiana* and *Rickettsia* sp. (Palomar et al., 2012). It appears that differences in co-infection

190 found in *I. ricinus* depend on the local epidemiology of infectious diseases and the availability  
191 of host reservoirs of those bacteria.

192 Co-infections by tick-borne pathogens in humans have been reported. These occurred  
193 from the bite of a single tick infected by several pathogens, or repetitive bites of several  
194 infected ticks (Diuk-Wasser et al., 2016; Krause, 2002). Co-infections in humans mostly  
195 involved Lyme borreliosis with either human anaplasmosis or babesiosis (Swanson et al.,  
196 2006). However, in Europe, co-infection with *Borrelia* spp. , *A. phagocytophilum* and *Babesia*  
197 spp. was observed in patients with tick-borne encephalitis in Poland (Moniuszko et al., 2014).  
198 Co-infection with multiple pathogens may lead to an increase in the severity of the disease  
199 and the development of an uncharacterized illness (Stanek et al., 2012). The risk of infections  
200 in people with a tick-borne pathogen other than Lyme borreliosis or tick-borne encephalitis  
201 after a bite from a co-infected tick is 2.5% (Hofhuis et al., 2017; Jahfari et al., 2016).

202 In the present study, we used MALDI-TOF MS to confirm the morphological  
203 identification of ticks we tested. Applying this technology, 68% (34/50) of ticks, for all of  
204 which a high-quality spectrum had been obtained, were successfully identified as *I. ricinus*.  
205 This innovative proteomic approach was successfully applied in identifying laboratory-reared  
206 ticks using only legs (Rothen et al., 2016; Yssouf et al., 2013), as well as ticks collected in the  
207 field (Kumsa et al., 2016). However, for some specimens, low-quality spectra were obtained.  
208 This may be due to several reasons including the storage method, homogenization method or  
209 the size of the nymph legs which are smaller than adult legs.

210 In conclusion, this study has led to the identification of several zoonotic pathogens  
211 including an uncharacterized *Bartonella* sp. carried by *I. ricinus* nymphs in the region of  
212 Alsace, northern France. A high level of co-infection was revealed in the ticks collected in  
213 this area, despite the small number of tested specimens. Infection or co-infection by multiple-

214 organisms suggests the need to increase our vigilance to avoid tick bites, especially by  
215 nymphs which are more difficult to detect than adults.

216

217 **Competing interests**

218 The authors declare that they have no competing interests

219

220 **Author contribution**

221 Conceived and designed the experiments: NA, AL, PP. Performed the experiments: NA.

222 Analyzed the data: NA, AL, PP. Contributed reagents/materials/analysis tools: NB, DR, HD,

223 OM. Drafted the paper: NA. Critically reviewed the paper: PP, AL, NB, BJ.

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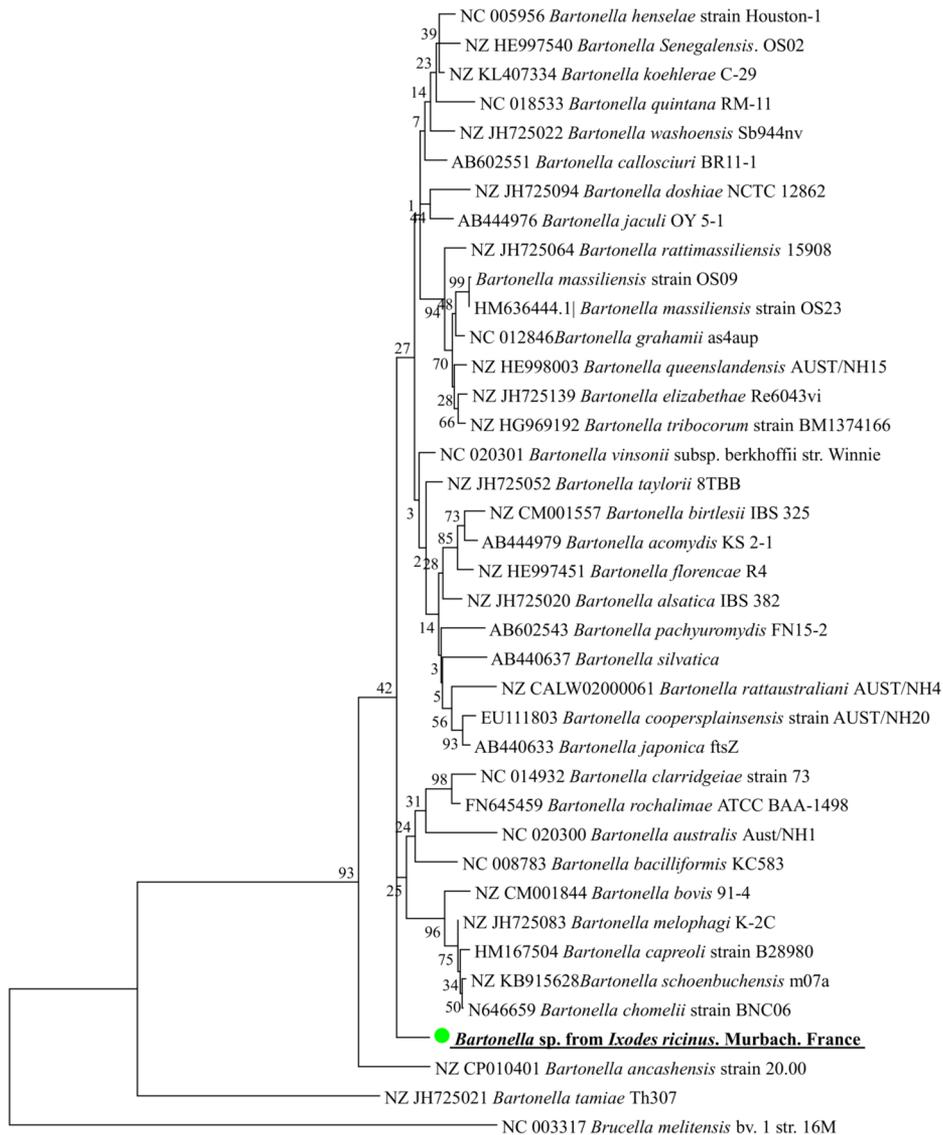
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364 **Figure legends**

365 **Figure 1. Phylogenetic tree showing the position of *Bartonella* sp. amplified from**  
366 ***I. ricinus* in this study compared to other species.** The sequences of the *Bartonella* sp.  
367 obtained in this study were aligned with *Bartonellaceae* family sequences available on  
368 GenBank using CLUSTALW implemented on BioEdit v3 (Hall, 1999). The sequence of *ftsZ*  
369 gene was first aligned, gaps and missing data were eliminated. The phylogenetic tree was  
370 constructed using MEGA7 (Kumar et al., 2016). The evolutionary history was inferred by  
371 using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The  
372 percentage of trees in which the associated taxa clustered together is shown next to the  
373 branches. Initial tree(s) for the heuristic search were obtained automatically by applying  
374 Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the  
375 Maximum Composite Likelihood (MCL) approach and then selecting the topology with  
376 superior log likelihood value. A discrete Gamma distribution was used to model evolutionary  
377 rate differences among sites (4 categories (+G, parameter = 0.3117)). The tree is drawn to  
378 scale, with branch lengths measured in the number of substitutions per site. The analysis  
379 involved 39 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.  
380 All positions containing gaps and missing data were eliminated. There was a total of 335  
381 positions in the final dataset.

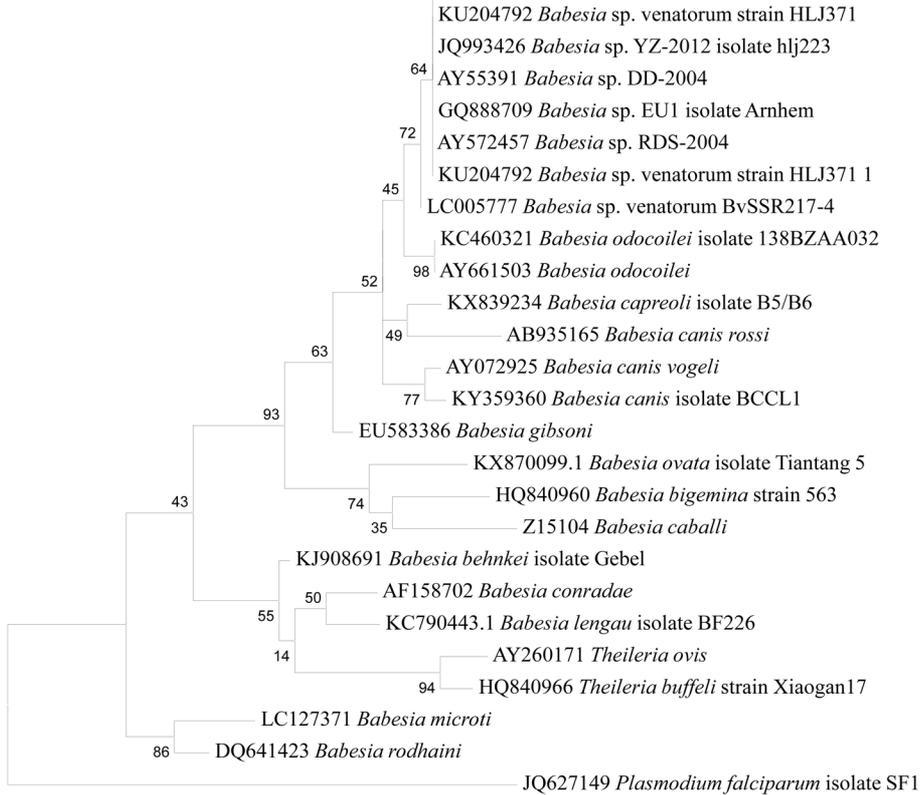
382 **Figure 2. Phylogenetic tree showing the position of *Babesia* sp. amplified from *I. ricinus***  
383 **in this study compared to other species.** The sequence of the *Babesia* sp. obtained in this  
384 study was aligned with *Babesiidae* family sequences available on GenBank using  
385 CLUSTALW implemented on BioEdit v3 (Hall, 1999). The sequence of *18S* gene was first  
386 aligned, gaps and missing data were eliminated. The phylogenetic tree was constructed using  
387 MEGA7 (Kumar et al. 2016). The evolutionary history was inferred by using the Maximum

388 Likelihood method based on the Hasegawa-Kishino-Yano model. The tree with the highest  
389 log likelihood (-1164.9283) is shown. The percentage of trees in which the associated taxa  
390 clustered together is shown next to the branches. Initial tree(s) for the heuristic search were  
391 obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of  
392 pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach,  
393 and then selecting the topology with superior log likelihood value. The tree is drawn to scale,  
394 with branch lengths measured in the number of substitutions per site. The analysis involved  
395 26 nucleotide sequences. All positions containing gaps and missing data were eliminated.  
396 There were a total of 192 positions in the final dataset.



0.2

● **Babesia sp. from Ixodes ricinus . Murbach. France**



0.050