



HAL
open science

**Characterization and diversity of Babesia sp. YLG, a
new member of the Peircei group infecting
Mediterranean yellow-legged gulls (*Larus michahellis*)**
Claire Bonsergent, Marion Vittecoq, Carole Leray, Louisiane Burkart, Karen
Mccoy, Laurence Malandrin

► **To cite this version:**

Claire Bonsergent, Marion Vittecoq, Carole Leray, Louisiane Burkart, Karen Mccoy, et al.. Characterization and diversity of Babesia sp. YLG, a new member of the Peircei group infecting Mediterranean yellow-legged gulls (*Larus michahellis*). *Ticks and Tick-borne Diseases*, 2022, 13 (1), pp.101852. 10.1016/j.ttbdis.2021.101852 . hal-03388445

HAL Id: hal-03388445

<https://hal.science/hal-03388445>

Submitted on 20 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Characterization and diversity of *Babesia* sp. YLG, a new member of the Peircei group**
2 **infecting Mediterranean yellow-legged gulls (*Larus michahellis*)**

3
4
5
6
7
8
9
10 5 Claire Bonsergent¹, Marion Vittecoq^{2,3}, Carole Leray³, Louisiane Burkart³, Karen D. McCoy^{2,4},
11
12 6 Laurence Malandrin^{1*}
13
14
15
16

17 8 1. INRAE, Oniris, BIOEPAR, 44300, Nantes, France

18
19 9 2. Centre for Research on the Ecology and Evolution of Diseases (CREES),
20
21 Montpellier, France
22 10

23
24 11 3. Tour du Valat, Research Institute for the Conservation of Mediterranean Wetlands,
25
26 Arles, France
27 12

28
29 13 4. MIVEGEC, University of Montpellier CNRS IRD, Centre IRD, Montpellier, France
30
31
32 14
33
34 15
35
36

37 16 Corresponding author: Laurence Malandrin. Oniris Ecole Nationale Vétérinaire, Alimentaire et
38
39 de l'Alimentation Nantes Atlantique. UMR INRAE/Oniris BIOEPAR. 101 route de Gachet.
40 17
41 CS40706. 44 307 Nantes cedex 3. France. laurence.malandrin@oniris-nantes.fr. +(33) 2 40 68
42 18
43 78 57.
44 19
45
46
47 20
48
49
50 21
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

22 **Abstract**

23
24 Avian infecting piroplasms are largely under-studied compared to other hemoparasites, and this
25 paucity of information has blurred our phylogenetic and biological comprehension of this
26 important group as a whole. In the present study, we detected and characterized *Babesia* from
27 yellow-legged gull (*Larus michahellis*) chicks from a colony in southern France. Based on
28 morphological and molecular characterizations, a new *Babesia* species belonging to the Peircei
29 group, a clade of avian-specific piroplasms, was identified. Due to the complexity of species
30 delineations and the low number of parasites characterized in this clade to date, a species name
31 was not yet attributed; we refer to it for now as *Babesia* sp. YLG (Yellow-Legged Gull). High
32 prevalence (85% and 58% in 2019 and 2020, respectively) and high parasitemia (up to 20% of
33 parasitized erythrocytes) were recorded in chicks, without any obvious clinical signs of
34 infection. Although the 16 isolates had identical 18S rRNA gene sequences, 6 genetic variants
35 were described based on partial *cox1* sequencing, with evidence of chicks co-infected by two
36 variants. Transmission of *Babesia* sp. YLG via the soft tick *Ornithodoros maritimus* is
37 discussed.

38

39 **Keywords**

40 *Babesia*, *Larus michahellis*, Peircei group, molecular characterization, 18S rRNA gene, *cox1*

41

42 **Introduction**

43

44 Hemoparasite infections have been found to occur in almost one third (27%) of all seabird
45 species (Quillfeldt et al., 2011) and in over 40% of species examined in rehabilitation centers
46 (Parsons et al., 2017). Although some infections are considered to impact individual bird health
47 (Parsons et al., 2017; 2018), little is known about how these parasites may affect seabird
48 population dynamics and conservation. Hemoparasites use different vectors to achieve
49 transmission (ticks, fleas, biting midges, flies, mosquitoes) and their contact with birds will
50 depend on avian habitat use and lifestyle. Colonial seabirds may be particularly exposed to
51 these vectors during reproduction when they come to land to breed. Indeed, because of their
52 tendency to breed in high densities and reuse the same colony locations, vectors may become
53 very abundant locally. For example, large populations of nest-dwelling ticks are known to
54 build-up in seabird breeding colonies reused over long periods of time (Danchin, 1992; McCoy
55 et al., 2016). Among hemoparasites, tick-transmitted piroplasms are extremely common in
56 mammals and known to cause severe disease of great medical and veterinary importance
57 (Homer et al., 2000; Schnittger et al., 2012). In birds, piroplasms are poorly described,
58 especially compared to other hemoparasites such as *Plasmodium*, *Haemaphysalis*, *Hepatozoon*
59 and *Leucocytozoon* (Peirce, 2005). Interestingly, most cases of infection to date have been
60 reported in seabirds.

61

62 Piroplasm infections in birds are due to *Babesia* species, but most of the 16 described species
63 to date have been defined based solely on morphological descriptions and/or the observed hosts
64 (Peirce, 2000; 2005; Yabsley et al., 2017). Initial molecular characterizations and phylogenetic
65 analyses have demonstrated that avian *Babesia* spp. do not form a monophyletic group but

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

66 rather cluster into three distinct groups (Yabsley et al., 2017), belonging to two of the ten major
67 clades described for piroplasms (Clades V and X, Jalovecka et al., 2019). Clade X corresponds
68 to *Babesia sensu stricto* infecting a wide range of hosts, including birds. The two avian groups
69 described by Yabsley et al.,(2017), *Kiwiensis* and *Bennetti*, belong to this clade. In contrast,
70 the entire clade V seems to be largely restricted to seabird-infecting piroplasms, the *Percei*
71 group. Given the paucity of studies on seabird piroplasms, it is likely that diversity of these
72 groups are largely underestimated. The overall phylogeny of the *Babesiidae*, and our
73 understanding of its evolution, should therefore gain in accuracy, or even change, as new avian
74 *Babesia* sequences from additional hosts and locations are discovered.

75
76 *Babesia*-infected birds have been reported from different parts of the world (Europe, New
77 Zealand, Australia, South Africa, Brazil, USA, Pacific Islands, Singapore), infecting different
78 avian orders (Apterygiformes, Charadriiformes, Passeriformes, Pelicaniformes,
79 Sphenisciformes, Suliformes). The only *Babesia* reported from seabirds in Europe to date is *B.*
80 *bennetti* described in the yellow-legged gull (*Larus michahellis*) on Benidorm island, Spain
81 (Mediterranean Sea) (Merino, 1998). In a recent study, *Ornithodoros maritimus* ticks
82 (Argasidae) collected in a Yellow-legged gull *Larus michahellis* colony in the south of France
83 were found to carry a *Babesia* sp. In this study, detection was performed using a real-time PCR
84 micro-array assay designed for mammalian *Babesia* and species identifications could not be
85 made (Dupraz et al., 2017). The present study aimed to verify *Babesia* species infection in the
86 birds from this colony, characterize the *Babesia* species implicated and better evaluate its
87 prevalence and diversity within this seabird population.

88
89
90

91

92

93 **Material and methods**

94 **Study location**

95 Carteau island is a flat islet in the Gulf of Fos in the Camargue area of southern France
96 (43°22'39"N 4°51'28"E). A population of about 300 breeding pairs of yellow-legged gull
97 (*Larus michahellis*, *Laridae*, Charadriiformes) occupy the 1.36 km² islet during the
98 reproductive season (March to June).

99

100 **Blood samples from yellow-legged gull chicks**

101 Blood collection was performed in May and June 2019 from 20 yellow-legged gull chicks (3 to
102 4 weeks old), all from different nests. In spring 2020, 38 additional blood samples were
103 collected from 36 chicks coming from 21 nests and sampled at three different time points
104 (18/05, 26/05 and 02/06). Two chicks were sampled twice at a 2-week interval (18/05 and
105 02/06). The capture and manipulation of chicks was approved by the local animal ethical
106 committee (Apafis request 25183) and permission to access the Carteau colony was provided
107 by Grand Port Maritime de Marseille and the DDTM 13/Service Mer Eau Environnement/Pôle
108 Nature et Territoires (n°13-2018-02-2-003). All handled chicks were ringed under the CRBPO
109 Personal ringing program n°990 (Museum National de l'Histoire Naturelle, Paris).

110 Between 0.2 and 1ml of blood was obtained from the brachial vein of chicks using a 26G
111 0.45x12mm needle rinsed with heparin. In 2019 and 2020, blood smears were freshly prepared,
112 air-dried, fixed with methanol, and Giemsa stained. They were examined under light
113 microscopy using a 100 × oil immersion lens to estimate parasitemia levels and describe

114 morphological features. In 2019, 20 μ L of the blood samples were centrifuged and red blood
115 cells were kept frozen at -20°C until DNA extraction.

116 **Genomic DNA extraction from yellow-legged gull blood**

117 Samples of 10 μ L of frozen pelleted RBC (Red Blood Cells) from 20 *Larus michahellis* chicks
118 collected in 2019 were washed twice with 500 μ L of PBS1X. Genomic DNA was then extracted
119 using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's
120 instructions for ≤ 300 μ L samples. DNA was eluted with 100 μ L of elution buffer and was
121 stored at -20°C until use.

123 **Molecular detection of blood piroplasms**

124 An initial PCR was performed to amplify the entire 18S rRNA gene of the putative piroplasms
125 using CRYPTOOF and CRYPTOR primers (Table 1), from two blood samples found highly
126 infected on the blood smears. Reactions were carried out in 30 μ L reaction mixtures containing
127 1 X buffer, 4 mM MgCl_2 , 0.2 mM each dNTP (Eurobio), 1 unit GoTaq G2 Flexi DNA
128 Polymerase (Promega), 0.5 μ M of each primer and 10 μ L of DNA template. PCR cycling
129 comprised 5 min at 95°C , 40 cycles at 95°C for 30s, 30s at 63°C , 1 min at 72°C , and a final
130 extension at 72°C for 5 min. A nested PCR using BAB-GF2 and BAB-GR2 primers was
131 conducted with 10 μ L of 1/40 diluted amplicons in a 30 μ L reaction mixture containing the
132 same components as the first PCR (Table 1). Cycling conditions were the same as the primary
133 reaction except the annealing temperature was 61°C . Amplified fragments were purified with
134 ExoSAP-IT reagent following manufacturer's recommendations (Affymetrix) and were
135 sequenced bi-directionally using the same primers (Eurofins Genomics, Germany). Sequences
136 were then assembled using the Geneious R6 software (<https://www.geneious.com>) and an
137 online BLAST (National Center for Biotechnology Information) was performed.

138

1
2 **139 Molecular characterization of blood piroplasms**
3

4
5 **140 18S *rRNA* gene**
6

7 **141** As cross-amplification with *Larus michahellis* DNA was observed, new primers were designed
8
9 **142** to improve the specificity of our amplification of the Percei group *Babesia* (BLAST result),
10
11 **143** (Table 1). For this purpose, the 18S rRNA gene sequences of *Larus michahellis* (this study),
12
13
14 **144** and of *Babesia* spp. from the Peircei group, the Bennetti group and the Kiwiensis group (Table
15
16
17 **145** 2) (Yabsley et al., 2017) retrieved from the GenBank database
18
19 **146** (<http://www.ncbi.nlm.nih.gov/genbank>) were aligned with ClustalW (Thompson et al., 1997)
20
21
22 **147** (Supplementary data 1). The newly designed primers (18SBp_fw and 18SBp_rev) were used
23
24 **148** in a nested PCR (see reaction and cycling conditions above, with an annealing temperature at
25
26 **149** 60°C) to amplify an expected 1529 bp fragment after a primary amplification with CRYPTOOF
27
28
29 **150** and CRYPTOR primers (Table 1).
30

31 **151**

32
33
34 **152 *Cox1* gene**
35

36 **153** *Cox1* (cytochrome oxidase subunit 1) sequences of piroplasms from different phylogenetic
37
38
39 **154** groups were aligned in order to design primers that would specifically amplify the *cox1*
40
41 **155** sequence of the yellow-legged gull parasites (Table 1, Supplementary data 2). These primers
42
43 **156** (COX1_fw and COX1_rev) enabled the amplification of a 1220 bp *cox1* fragment (see reaction
44
45
46 **157** and cycling conditions above, with an annealing temperature at 54°C).
47

48 **158**

49
50
51 **159** Amplified fragments of 18S *rRNA* and *cox1* genes were purified, sequenced and analyzed as
52
53 **160** described above.
54

55
56 **161**

57
58 **162 Phylogenetic analysis**
59
60
61
62
63
64
65

163 Alignments of sequences obtained from the *Babesia* isolates from *Larus michahellis* with other
164 avian and mammalian piroplasms' sequences retrieved from GenBank were generated. For the
165 18S rRNA gene, the multiple sequence alignment ClustalW program was used and sequences
166 were trimmed with the Gblocks method (Castresana, 2000), allowing small final blocks. For
167 the *cox1* gene, sequences were aligned with the multiple sequence alignment MUSCLE
168 program (Edgar, 2004). Phylogenetic analyses used trimmed sequences of 1291 bp for the 18S
169 rRNA and 951 bp for *cox1* genes. Maximum likelihood phylogenetic trees were produced using
170 MEGA version X (Kumar et al., 2018) with 1000 bootstrap replications, based on the Tamura-
171 Nei + I + Γ (Tamura and Nei, 1993) for the 18S rRNA gene and based on the GTR + G
172 + Γ (Tavaré, 1986) model for the *cox1* gene. The appropriate model of nucleotide substitution
173 for ML analysis was selected based on the Bayesian Information Criterion (BIC) computed by
174 MEGA-X.

176 **GenBank deposition**

177 Nucleotidic sequences obtained in this study were submitted to Genbank with accession
178 numbers MZ541058 and MZ558162-MZ558167 for 18S rRNA gene and *cox1* variants
179 sequences of *Babesia* sp. YLG isolates respectively.

182

1
2 **183 Results**

3
4
5 **184 Prevalence and parasitemia of intraerythrocytic piroplasms**

6
7 185 In 16 of 20 blood smears of tested chicks (80%) analyzed in 2019, intraerythrocytic pale,
8
9 186 rounded or ameboid parasitic forms were detected, with parasitemia ranging from 0.1%
10
11 187 (estimation made on 20 fields of about 180 erythrocytes) to 15% of erythrocytes (295 infected
12
13 188 Red Blood Cells iRBC/1994 RBC). Parasite DNA was successfully amplified from blood of all
14
15 189 positive smear samples using the *Babesia/Theileria* broad range primers. Blood from one blood
16
17 189 smear negative sample of a single chick was also found positive by PCR (prevalence of
18
19 190 infection increased to 85%) (Table 3). Further molecular characterization was performed using
20
21 191 these 17 blood samples (see below).
22
23
24 192

25
26 193 To examine the local maintenance of these parasites on Carteau, 38 additional blood smears
27
28 194 were prepared from blood of the 36 chicks sampled in May and June 2020. Their observation
29
30 195 revealed that 58% of chicks carried parasites morphologically identical to those described in
31
32 196 2019, with the same range of parasitemia (infection in 0.1% to 15% of erythrocytes).
33
34 196

35
36 197 Most chicks had parasitemia above 1% both years: 59% of the infected chicks in 2019 (10/17)
37
38 198 and 75% of the infected chicks in 2020 (15/20). Highly parasitized chicks with parasitemia
39
40 199 above 10% also occurred (7 chicks in 2019 and 5 chicks in 2020). Of the two chicks sampled
41
42 200 twice, one was found negative on the first smear and the other weakly infected (<0.1%
43
44 201 parasitemia). Two weeks later, they were both highly parasitized (>10% parasitemia).
45
46 201

47
48 202

49
50
51 **203 Morphological description of the avian piroplasm (Figure 1)**

52
53 204 The most striking feature of the blood smears was the presence of large, pale, rounded,
54
55 205 amoeboid, pleomorphic forms within the erythrocytes. On all observed smears, they
56
57 206 represented 80-90 % of the intraerythrocytic forms (n = 441/527 iRBC, of 4026 RBC analyzed)
58
59
60
61
62
63
64
65

207 (Figure 1). Amoeboid forms the length of the host cell nucleus could be observed alongside the
208 nucleus or folded at one cell pole (Figure 1 A, B, C, E). Within RBCs, parasites could be found
209 either at a polar position (43%, n = 190/441 iRBC) or alongside the host nuclei (57%, n =
210 251/441 iRBC). Despite occupying up to one fourth of the cytoplasm, host cell nuclei were
211 never displaced by the parasite (Figure 1 B).

212 In the case of heavy infection (parasitemia above 10%), two parasites per host cell were frequent
213 (6%, n = 33/527 iRBC), sometimes three (2%, n = 10/527 iRBC), of similar or different sizes
214 and stages (Figure 1 D).

215 The different forms occurring in iRBCs could represent different life stages of the parasites -
216 trophozoites and schizonts. In the smallest trophozoites, chromatin occupied a large proportion
217 of the cell (Figure 1 G). With increasing trophozoite size, the cytoplasm of the parasite occupied
218 almost the whole cell, with the chromatin displaced to the periphery, with a dotted or uneven
219 local distribution (Figure 1 A, C and E). Chromatin then condensed into two to four dots
220 budding from the remaining rounded or amoeboid cytoplasm, developing into extremely
221 various schizont forms: fan-shaped, cruciform, “cow’s udder” shapes with chromatin dots at
222 each extremities (Figure 1 A,C, G, H) (4% of the observed intra-erythrocytic parasites, n =
223 22/527 iRBC). Nuclei of the future merozoites were mostly seen in the mean body or at the
224 distal pole of the schizont, opposite of the amoeboid chromatin-free cytoplasm remaining at the
225 basal region of the four developing merozoites (Figure 1 C and G). Very few erythrocytic cells
226 with 4 identically-elongated merozoites with central nuclei (4%, n = 21/527 iRBC) were found
227 (Figure 1 C, D and F).

228
229

230 **Molecular characterization of the yellow-legged gull piroplasm**

231 **Analysis of the 18S rRNA gene**

232 The first amplification of partial 18S rRNA genes on two positive samples using the
233 *Babesia/Theileria* broad range primers CRYPTOR/CRYPTOF and GF2/GR2 resulted in
234 sequences of 552 bp. Blastn analysis of the longest amplicon revealed an identity above 96,13%
235 with different avian piroplasm species, all belonging to the Peircei *Babesia* group.
236 Amplification was then obtained for 15 more blood samples, and all sequences were identical.
237 The specific nested PCR using the internal primers designed to amplify the different species of
238 the Peircei *Babesia* group enabled us to obtain a longer fragment of the 18S rDNA gene for 16
239 individuals (1529 bp), which was then sequenced bidirectionally. Identical partial 18S rRNA
240 gene sequences (1461-1485 bp) were again found.

241 Blastn analysis of this sequence revealed high identities with several Peircei *Babesia* sequences
242 from infected marine birds. The highest identities were obtained with a *B. poelea* sequence from
243 brown boobies (*Sula leucogaster*) in the central Pacific (DQ2000887, 99.52%) and a *Babesia*
244 sp. from masked and brown boobies (*Sula dactylatra* and *S. leucogaster*) from Brazilian
245 offshore islands (KC754965, 99.44%). The other sequences with identities over 99% belonged
246 to *B. peircei* (99.32%), *B. uriae* (99.25%) and *B. ugwiensis* (99.18%) (refer to Table 2 for a
247 detailed description of hosts and their geographic origin).

248 The unique sequence from this study was aligned using ClustalW with the longest available
249 sequences of representative *Babesia* belonging to the Peircei group. Single nucleotide
250 polymorphisms among these sequences are highlighted in Figure 2. The sequence from the
251 yellow-legged gull *Babesia* differs by 7 nucleotide exchanges from *B. poelea* (DQ2000887,
252 *Sula leucogaster*, Central Pacific) and 8 nucleotide exchanges from *Babesia* sp. 1 JM-2013
253 (KC754965, *Sula leucogaster* and *S. dactylatra*, Brazil), while the two latter sequences differ
254 at only 3 positions (on a gene length of 1426 bp). Four nucleotide exchanges are unique to the

255 sequence identified in the present study (highlighted in red in Figure 2). The number of
1 polymorphic sites increased to 10 when compared to *B. peircei* (MF288025, *Spheniscus*
2 256 *demersus*, South Africa), 12 with *B. ugwiensis* (MF288027, *Phalacrocorax capensis* and *P.*
3 257 *neglectus*, South Africa) and 13 with *B. uriae* (FJ717705, *Uria aalge*, USA California). While
4 258 6 positions differ between *B. peircei* and *B. ugwiensis*, they both show 12 nucleotide
5 259 exchanges with the *B. uriae* sequence. With 18 and 20 nucleotide exchanges, the *Babesia* from
6 260 France differs markedly from *Babesia* sequences characterized in seabirds of the eastern south
7 261 pacific, from *Eudyptula minor* in Australia (KP144322) and the two *B. poelea*-like identified
8 262 from different seabirds in New Zealand (represented by MF162302 and MF162305) (refer to
9 263 Table 2 for detailed descriptions of the aforementioned isolates).
10 264
11 265

266 **Analysis of the *cox1* gene**

267 Partial *cox1* sequences (1172 bp) were obtained from 9 blood samples. Blastn analysis revealed
268 the highest identity (84.69%) with the single *cox1* sequence available for the Peircei group,
269 from *B. ardeae* detected in a grey heron (*Ardea cinerea*) in Singapore (MH457241.1). The nine
270 *cox1* sequences were not identical, and polymorphism was detected at 5 positions in the partial
271 gene sequences (Table 4). Four genetic variants differed by a combination of one or two SNPs
272 at 4 positions (89, 107, 860 and 917). The most abundant variant, named *cox1a*, was detected
273 in 3 blood samples, the three others (variants *cox1b*, *c* and *d*) were unique (Table 4). In three
274 chicks, chromatograms with double peaks of identical intensities were detected. In two of them
275 (EA773745 and EA773911), the positions of the double peaks corresponded to variable
276 positions already identified in variants *cox1a* and *cox1b* (107 and 917) with the same
277 nucleotides (A or G at position 107 and C or T at position 917). In chick EA677439, one double
278 peak occurred at a position not yet described as polymorphic in the former sequences
279 (possibility of a G at position 1123). The presence of two variants within the same chick would

280 explain these double peaks, resulting from the co-infections with already detected variants (a
281 and b in chicks EA773911) or new variants (variants e in chick EA773745 and f in chick
282 EA677439). At least six genetic variants could therefore be present in the yellow-legged gull
283 chicks present on the islet of Carteau, the variant *cox1a* being the most abundant (Table 4).
284 These nucleotide exchanges are synonymous, except at position 1123 resulting in an amino acid
285 modification from phenylalanine to cysteine.

286

287 **Phylogenetic analyses**

288 The 18S rRNA (Figure 3) and *cox1* (Figure 4) phylogenetic analyses confirmed the position of
289 the *Babesia* sp. YLG into the Peircei clade, with strong bootstrap values of 100 and 99,
290 respectively. According to the 18S rRNA phylogenetic analysis, *B. ardeae* detected in the grey
291 heron represents a sister taxon to a clade comprised of remaining *Babesia* spp. On one hand,
292 this clade of remaining *Babesia* spp. splits into a subclade of *Babesia* spp. isolated from sea
293 birds in South Africa, America and Europe supported with a moderate bootstrap of 68, and, on
294 the other hand, into a strongly supported subclade (bootstrap 99) of *Babesia* spp. isolated from
295 sea birds from the Eastern south pacific region.

296

1
2 **297 Discussion**

3
4
5 298

6
7 299 In the present study, we characterized a piroplasm in the blood of yellow-legged gulls from
8
9 300 southern France. Morphological descriptions based on 37 blood smears, molecular
10
11 301 characterization (partial 18S rRNA gene sequences from 17 different isolates and partial *cox1*
12
13 302 sequences from 9 isolates) and a phylogenetic analysis places this *Babesia* into the Peircei
14
15
16 303 clade. This group is mainly composed of seabird-infecting piroplasms, *B. ardeae* from the grey
17
18 304 heron being the only current exception (Chavatte et al., 2017). *B. ardeae* differs from the other
19
20
21 305 members of the Peircei group by 32 unique polymorphic sites (i.e., not shared by any other
22
23 306 member of the Peircei group) on the 18S rRNA gene (nt 171-1630), while *B. poelea*, *B.*
24
25
26 307 *peircei*/*B. ugwidiensis*, *B. uriae* and the austral members of the group (South Australia and New
27
28 308 Zealand) have only 1, 2, 5 and 11 unique polymorphic sites, respectively. *B. ardeae* places as
29
30
31 309 sister to other *Babesia* species of the Peircei group on phylogenetic trees (Yabsley et al., 2017;
32
33 310 this study), demonstrating it is more distantly related to other members of the Peircei clade.

34
35
36 311

37
38 312 Morphologically, the yellow-legged gull parasite resembles *B. poelea* described in brown
39
40 313 boobies (*Sula leucogaster*) from the Johnston Atoll, Central Pacific (Work and Rameyer, 1997):
41
42
43 314 high proportion of large amoeboid forms (80%), no displacement of the host nucleus,
44
45 315 pleomorphic dividing forms with chromatin granules either in the mid-body or near the blunt
46
47
48 316 end of the developing merozoites. The chromatin position in the elongated merozoites is a
49
50 317 feature used to differentiate *B. peircei* (middle or distal part) from *B. ugwidiensis* (proximal
51
52 318 part) (Yabsley et al., 2017). In *B. peircei*, the position of the trophozoites was mostly polar in
53
54
55 319 red blood cells (Earlé et al., 1993), while no preferential position could be observed for *B.*
56
57
58 320 *poelea* or for the gull parasite. Morphology as a single criterion should be considered with
59
60 321 extreme caution, as the same parasite species may show different morphologies according to

322 the host it's infecting, a phenomenon known as pleomorphism (Schnittger et al., 2012). This
323 was well-described for *B. divergens* developing in ox, gerbil and human (Gorenflot et al., 1991).
324
325 In the case of the gull parasite, molecular analyses corroborate the morphological resemblance
326 to *B. poelea*, as they share the highest identity for the 18S rRNA gene fragment (99.52%),
327 differing by only 7 nucleotide exchanges. The initial morphological description of *B. poelea*
328 (Work and Rameyer, 1997) was completed by the molecular characterization of the described
329 parasite from frozen blood of three highly infected birds (Yabsley et al., 2006). Another
330 molecularly-related *Babesia* (99.44% identity) was characterized from masked and brown
331 boobies (*Sula dactylatra* and *S. leucogaster*) sampled on Brazilian offshore islands, but their
332 morphology was not described (Quillfeldt et al., 2014). In South Africa, *Babesia* parasites from
333 Cape gannets (*Morus capensis*) were assumed to belong to *B. poelea* based on the host family
334 (Sulidae, Suliformes) with no complementary molecular or morphological characterizations
335 (Parsons et al., 2017). Two closely related parasites (2 nucleotide exchanges on the 18S rRNA
336 gene sequences) were characterized in New Zealand from Australasian gannets (*Morus*
337 *serrator*, Sulidae), red-billed gulls (*Chroicocephalus scopulinus*, Laridae, Charadriiformes) and
338 white-fronted terns (*Sterna striata*, Laridae) and named *B. poelea*-like I and II (Paparini et al.,
339 2014). However, these isolates are quite distant from *B. poelea* (98.96% identity) and form a
340 separate cluster on phylogenetic trees (Yabsley et al., 2017; this study). Their morphological
341 features were not described.

342
343 Confronted with the still uncertain species delineation within this group, we decided not to
344 name the *Babesia* described here from the yellow-legged gull, awaiting a more comprehensive
345 analysis based on molecular characterizations of other newly described seabird *Babesia*. The
346 parasite we describe here will therefore be referred to as *Babesia* sp. YLG (Yellow-Legged

347 Gull). *Babesia* sp. YLG is indeed more genetically distant from *B. poelea* (8 nucleotide
1 exchanges) than *B. peircei* is from *B. ugwidiensis* (6 nucleotide exchanges on the same 1426
2 bp fragment of the 18S rRNA gene) (Figure 3). The two latter species were described and
3
4
5 349 named based on morphology and host species (Earlé et al., 1997; Peirce and Parsons, 2012). As
6
7 350 with morphology, species delineations based solely on host specificity should be taken with
8
9 351 caution. For example, *B. poelea*-like I and II have been characterized in birds from different
10
11 352 avian orders (Charadriiformes and Suliformes) that share the same environment. They are more
12
13 353 than 99% identical at the 18S rRNA gene to an isolate from little penguins (Sphenisciformes)
14
15 354 (Paparini et al., 2014; Vanstreels et al., 2015) coming from a nearby region (southeastern
16
17 355 Australia/Tasmania versus New Zealand). However, neither of these two studies provided
18
19 356 morphological descriptions of the parasites in question. *B. bennetti* is the only previously
20
21 357 described *Babesia* infecting yellow-legged gulls in the Mediterranean Sea (Benidorm Island,
22
23 358 Spain: Merino 1998, Esparza et al, 2004). Despite morphological similarity with *Babesia* sp.
24
25 359 YLG, molecular analyses of *B. bennetti* (Criado et al., 2006) place the two parasites in separate
26
27 360 and very distant phylogenetic clades (Figure 3).
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

362
363 Based on previously published data, *Babesia* from the Peircei clade can probably be separated
364 into four phylogenetic groups: the *B. ardeae* group (Pelecaniformes), the *B. uriae* group
365 (Charadriiformes), the *B. poelea/B. peircei/B. ugwidiensis* group (Suliformes, Charadriiformes,
366 Sphenisciformes) and the austral group (Suliformes, Charadriiformes, Sphenisciformes). On
367 the basis of Schnittger's rules, all the parasites described in the Peircei group would be
368 considered a unique species as all sequences have a 18S rRNA gene identity of >98.5%
369 (threshold defined by Schnittger is 97.9%) (Schnittger et al., 2003). Only *B. ardeae* could be
370 considered as a separate species. However, the tempo of evolutionary change in the Peircei
371 group might derived from that of *Babesia/Theileria* sensu stricto groups from which these rules

372 were defined. For now, it is difficult to assess this possibility due to the paucity of publications
1
2 373 and genetic data (intraspecific and interspecific) available for this group.
3

4
5 374

6
7 375 Intraspecific diversity has seldomly been addressed in studies on seabird *Babesia*. When 18S
8

9 376 rRNA gene sequences were obtained from several individuals within a study area, they were
10

11 377 usually conserved (Quillfeldt et al., 2014; Vanstreels et al., 2015; Yabsley et al., 2017). This
12
13

14 378 was also the case for the 17 different chick isolates characterized in the present study. However,
15

16
17 379 slight variations (2 nucleotide exchanges) were found between the *B. poelea*-like I and II from
18

19 380 New Zealand. Intraspecific variability was analyzed for *B. poelea*, *B. peircei* and *B. ugwidiensis*
20

21 381 using ribosomal intergenic regions ITS1 and ITS2. No variability was observed for *B. poelea*
22
23

24 382 from two samples collected within the same colony (Yabsley et al., 2006). On the contrary,
25

26 383 intraspecific diversity was noted in both intergenic regions for *B. peircei* from penguins and *B.*
27

28
29 384 *ugwidiensis* from Cape and Bank Cormorants, with no precision from where birds originated
30

31 385 (Yabsley et al., 2017). In the phylogenetic analyses, *B. ugwidiensis* ITS sequences clustered
32
33

34 386 together regardless of the host species of origin, and were well-separated from *B. peircei*
35

36 387 sequences from penguins. In our study, a low level of sequence variability of *Babesia* sp. YLG
37

38
39 388 was observed at the *cox1* gene sequenced from nine chicks sampled from the same colony and
40

41 389 year. Based on 5 SNPs, six genetic variants were identified, differing from one another by one
42

43 390 or two SNPs. Three out of the nine birds tested seemed to be infected by two different variants,
44
45

46 391 indicating either multiple transmissions or transmission by co-infected vectors. The next step
47

48 392 will now be to sequence all currently described avian *Babesia* species using the same genes and
49

50
51 393 near complete sequences to evaluate general levels of intraspecific diversity in these parasites.
52

53 394
54

55
56 395 Ticks are vectors of piroplasmids, with transmission modalities varying according the
57

58 396 piroplasm group (Jalovecka et al., 2018). Several tick species, belonging to both the Ixodidae
59
60
61
62
63
64
65

397 (hard ticks) and the Argasidae (soft ticks) families, have been proposed to transmit *Babesia* to
398 different seabird species. *Ixodes uriae* and *Ornithodoros capensis* or *O. maritimus* are often
399 cited as putative vectors (Yabsley et al., 2009; 2017; Quillfeldt et al., 2014; Papparini et al., 2014;
400 Vanstreels et al., 2015; Chavatte et al., 2017). Ticks as a group encompass a wide range of
401 lifestyle strategies that ensure their ability to find a host and survive off-host periods. For
402 example, hard ticks frequently quest for hosts or use ambush strategies, waiting on vegetation
403 for appropriate hosts to pass by. In contrast, some species or immature stages are endophilic or
404 nidicolous, remaining close to areas that hosts regularly use, such as nests or resting sites. It is
405 this latter strategy that has been adopted by most soft tick species. For ticks exploiting seabirds,
406 almost all are nidicolous (Dietrich et al., 2011). Therefore, the transmission of parasites to
407 seabirds will be largely restricted to the breeding period when these birds spend a sufficiently
408 long period on land to favor exposure.

409
410 Several lines of evidence point towards a preferential role of argasid ticks in the transmission
411 of *Babesia* from the Peircei group to seabirds. 1- *Ornithodoros capensis* ticks (females and
412 nymphs) were found on a red-billed gull and *Babesia*-positive ticks were reported (Papparini et
413 al., 2014). 2- *O. capensis* larvae were also found on a *Phalacrocorax capensis* *Babesia*-free
414 chick which was a week later found to be infected with *B. ugwidiensis* (parasitemia 4%) (Peirce
415 and Parsons, 2012). 3- *Babesia* infections were found to be restricted to ground-nesting species
416 (masked and brown boobies) compared to tree or bush-nesting species (red-footed boobies and
417 black noddies) from the same sites which may be less favorable substrates for nidicolous ticks
418 (Quillfeldt et al., 2014). In the context of Carteau island, *Ornithodoros maritimus* (Argasidae)
419 is the only tick species recorded in yellow-legged gull nests (Dupraz et al., 2017; Rataud et al.,
420 2020). In a previous study, an undetermined *Babesia* species, probably *Babesia* sp. YLG, was
421 detected in 3% of *O. maritimus* adult ticks (6/201) collected from different nests in the colony

1 422 and screened using targeted PCR procedures developed for mammalian parasite species
2 423 (Dupraz et al., 2017). Experimental work and detailed analyses of infection in *Ornithodoros*
3
4 424 ticks will now be needed to conclude on the role of these ticks in the transmission of *Babesia*
5
6
7 425 parasites from the Percei group to seabirds.
8

9 426
10
11 427 In the present study, prevalence of infection by *Babesia* sp. YLG in yellow-legged gull chicks
12
13 428 was high (85% in 2019 and 58% in 2020), and parasitemia above 10% was frequently observed
14
15 429 (41% and 27% of the infected chicks in 2019 and 2020 respectively). Such results (high
16
17 430 prevalence and high parasitemia) are consistent with a nest-based exposure to infection. For
18
19 431 two chicks, *Babesia* sp. YLG multiplication was fulminant: initially found negative or with
20
21 432 almost undetectable parasite levels ($< 0.1\%$), parasitemia of 15-20% was recorded two weeks
22
23 433 later. These data suggest that chicks are infected in the nest and that high infection levels
24
25 434 develops as the chicks' immune system is maturing (Apanius, 1998). In many Percei infected
26
27 435 seabird species, prevalence was significantly higher in chicks and juveniles compared to adults
28
29 436 (Work and Rameyer, 1997; Quillfeldt et al., 2013; Parsons et al., 2017). Furthermore, in adult
30
31 437 birds, poor correspondence was also noted between blood smear detection and molecular
32
33 438 detection, most probably due to decreasing parasitemia concomitant with the acquisition of a
34
35 439 specific immunity post-exposure (Paparini et al., 2017). Even if adults are exposed to infected
36
37 440 ticks during the breeding period to the same extent as chicks, their immune status may enable
38
39 441 them to maintain parasitemia at a low and undetectable level, as is often the case for babesial
40
41 442 infections (Chauvin et al., 2009).
42
43
44
45
46
47
48
49
50

51 443
52
53 444 Despite parasitemia above 10% detected in about 1/3 of the sampled chicks, no clinical signs
54
55 445 of illness were noted in the field and chicks appeared in good health. However, juvenile Cape
56
57 446 cormorants admitted to a rehabilitation center in the Western Cape, South Africa, showed
58
59
60
61
62
63
64
65

1 447 significantly higher mortality compared to adults (Parsons et al., 2017). An effect of *Babesia*
2 448 infection was also reported in juvenile masked boobies, with infected individuals showing
3
4 449 lower body weights (Quillfeldt et al., 2014). Mortality in adult birds due to *Babesia*-infection
5
6
7 450 has been suggested to occur in King penguins (Parsons et al., 2018), indicating there may be
8
9
10 451 some interspecific differences in *Babesia* pathogenicity. In line with this hypothesis, the
11
12 452 yellow-legged gull chicks were reported to be significantly less immunocompetent compared
13
14 453 to storm petrel chicks, and therefore potentially more prone to *Babesia* infections (Esperza et
15
16
17 454 al., 2004). For now, it seems that *Babesia* infections in birds tend to be most commonly reported
18
19 455 from undernourished young individuals, when concomitant infections are present or when
20
21
22 456 individuals are otherwise compromised (Merino, 1998; Peirce, 2000). Under these conditions,
23
24 457 the relative impact of tick bites, *Babesia* infection and the potential presence of some other
25
26
27 458 pathogen on avian health cannot be independently evaluated and more direct observations of
28
29 459 infected birds are required to understand the degree of physiological stress imposed by *Babesia*
30
31 460 alone.

32
33
34 461
35
36 462 As highlighted by our current understanding of Peircei group of *Babesia*, there is a great
37
38
39 463 knowledge gap related to the identification, taxonomy, phylogeny, host specificity and
40
41 464 transmission ecology of avian *Babesia*. This gap is likely linked to both the logistical difficulty
42
43
44 465 in conducting research on these hosts (financial issues, accessibility, experimental host
45
46 466 infections and parasite transmission studies) and the fact that chronic infections of wildlife are
47
48
49 467 often overlooked when considering population dynamics and conservation issues. Furthermore,
50
51 468 the possible transmission of avian *Babesia* to humans or their domestic animals is likely limited
52
53
54 469 or absent reducing the possibilities to receive funding. We hope that future collaborative studies
55
56 470 will fill this knowledge gap, allowing us to better understand the full diversity of extant *Babesia*
57
58 471 species and the factors operating on the evolution of this group of important parasites.

472

1

2 473

3

4

5 474 Acknowledgements:

6

7 475 We thank all the people that assisted with field sampling : Marie Buysse, Elodie Conte, Thomas

8

9

10 476 Blanchon, Maxime Duhaydon, and Nathalie de la Cotte for blood smears mounting.

11

12 477

13

14 478 Funding:

15

16 479 Funding for this study was provided by the UMR BIOEPAR (TiBoDi team), the ANR grant

17

18 480 EcoDIS (ANR-20-CE34-0002) and by an exploratory research grant DISTIC from the Labex

19

20

21 481 CeMEB (Centre Méditerranéen de l'Environnement et de la Biodiversité) with the support an

22

23

24 482 ANR "Investissements d'avenir" program (ANR-10-LABX-04-01).

25

26

27 483

28

29 484 Conflict of interest: none.

30

31

32 485

33

34 486 Authors contribution:

35

36

37

38 487 **Claire Bonsergent:** Investigation, Formal analysis, Methodology, Visualization. **Marion**

39

40 488 **Vittecoq** Resources, Supervision, Investigation, Reviewing and Editing. **Carole Leray,**

41

42

43 489 **Louisiane Burkart** Resources. **Karen McCoy:** Resources, Conceptualization, Funding

44

45 490 acquisition, Reviewing and Editing. **Laurence Malandrin:** Funding acquisition,

46

47

48 491 Conceptualization, Supervision, Writing-Original draft preparation.

49

50

51

52 492

53

54

55 493

56

57 494 **References**

58

59 495

60

61

62

63

64

65

496 Apanius, V., 1998. Ontogeny of immune function. In: Avian Growth and Development –
1
2 497 Evolution Within the Altricial–Precocial Spectrum (eds Starck, J.M. & Ricklefs, R.E.).
3
4
5 498 Oxford University Press, Oxford, pp. 203–222.
6
7 499
8
9
10 500 Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in
11
12 501 phylogenetic analysis. Mol. Biol. Evol. 17, 540-552.
13
14 502 <https://doi.org/10.1093/oxfordjournals.molbev.a026334>.
15
16
17 503
18
19 504 Chauvin, A., Moreau, E., Bonnet, S., Plantard, O., Malandrin, L., 2009. *Babesia* and its hosts:
20
21 505 adaptation to long-lasting interactions as a way to achieve efficient transmission. Vet. Res.
22
23 506 40(2):37. <https://doi.org/10.1051/vetres/2009020>.
24
25
26 507
27
28
29 508 Chavatte, J.M., Okumura, C., Landau, I., 2017. Redescription of *Babesia ardeae* Toumanoff,
30
31 509 1940, a parasite of Ardeidae, including molecular characterization. Parasitol. Res. 116, 1089-
32
33 510 1097. <https://doi.org/10.1007/s00436-017-5394-1>.
34
35
36 511
37
38
39 512 Criado, A., Martinez, J., Buling, A., Barba, J.C., Merino, S., Jefferies, R., Irwin, P.J., 2006.
40
41 513 New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal
42
43 514 subunit and cytochrome b genes. Vet. Parasitol. 142(3-4), 238-247. [https://doi.org/](https://doi.org/10.1016/j.vetpar.2006.07.004)
44
45 515 [10.1016/j.vetpar.2006.07.004](https://doi.org/10.1016/j.vetpar.2006.07.004).
46
47
48 516
49
50
51 517 Danchin, E., 1992. The incidence of the tick parasite *Ixodes uriae* in kittiwake *Rissa*
52
53 518 *tridactyla* colonies in relation to the age of the colony and the mechanism of infecting new
54
55 519 colonies. Ibis 134, 134-141. <https://doi.org/10.1111/j.1474-919X.1992.tb08390.x>.
56
57
58 520
59
60
61
62
63
64
65

1 521 Dietrich, M., Gomez-Díaz, E., McCoy, K.D., 2011. Worldwide distribution and diversity of
2 522 seabird ticks: implications for the ecology and epidemiology of tickborne pathogens. Vector
3
4 523 Borne Zoonotic Dis. 11, 453-470. <https://doi.org/10.1089/vbz.2010.0009>.
5
6
7 524
8
9 525 Dupraz, M., Toty, C., Devillers, E., Blanchon, T., Elguero, E., Vittecoq, M., Moutailler, S.,
10
11 526 McCoy, K.D., 2017. Population structure of the soft tick *Ornithodoros maritimus* and its
12
13 527 associated infectious agents within a colony of its seabird host *Larus michahellis*. Int. J.
14
15 528 Parasitol. Parasites Wildl. 6(2), 122-130. <https://doi.org/10.1016/j.ijppaw.2017.05.001>.
16
17
18
19 529
20
21 530 Earlé, R.A., Huchzermeyer, F.W., Bennett, G.F., Brossy, J.J., 1993. *Babesia peircei* sp. nov.
22
23 531 from the Jackass Penguin. S. Afr. J. Zool. 28, 88-90.
24
25
26 532
27
28 533 Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high
29
30 534 throughput. Nucleic Acids Res. 32, 1792-97. <https://doi.org/10.1093/nar/gkh340>.
31
32
33 535
34
35 536 Esparza, B. Martinez-Abraín, A. Merino, S., Oro, D., 2004. Immunocompetence and the
36
37 537 prevalence of haematozoan parasites in two long-lived seabirds. Ornis Fennica, 81(1), 40-46.
38
39
40
41 538
42
43 539 Gorenflot, A., Brasseur, P., Precigout, E., L'Hostis, M., Marchand, A., & Schrevel, J., 1991.
44
45 540 Cytological and immunological responses to *Babesia divergens* in different hosts: ox, gerbil,
46
47 541 man. Parasitol. Res., 77(1), 3–12. <https://doi.org/10.1007/bf00934377>
48
49
50
51 542
52
53 543 Herwaldt, B.L., Cacciò, S., Gherlinzoni, F., Aspöck, H., Slemenda, S.B., Piccaluga, P.,
54
55 544 Martinelli, G., Edelhofer, R., Hollenstein, U., Poletti, G., Pampiglione, S., Löschenberger, K.,
56
57 545 Tura, S., Pieniazek, N.J., 2003. Molecular characterization of a non-*Babesia divergens*
58
59
60
61
62
63
64
65

546 organism causing zoonotic babesiosis in Europe. *Emerg. Infect. Dis.* 9(8), 942-8.
1
2 547 <https://doi.org/10.3201/eid0908.020748>.
3
4
5 548
6
7 549 Homer, M.J., Aguilar-Delfin, I., Telford 3rd, S.R., Krause P.J., Persing D.H., 2000. Babesiosis.
8
9 550 *Clin. Microbiol. Rev.* 13:451-69. <https://doi.org/10.1128/CMR.13.3.451>.
10
11 551
12
13 552 Jalovecka, M., Hajdusek, O., Sojka, D., Kopacek, P., Malandrin, L., 2018. The complexity of
14
15 553 Piroplasms life cycles. *Front. Cell. Infect. Microbiol.* 8:e248.
16
17 554 <https://doi.org/10.3389/fcimb.2018.00248>.
18
19 555
20
21 556 Jalovecka, M., Sojka, D., Ascencio, M., Schnittger, L., 2019. *Babesia* Life Cycle - When
22
23 557 Phylogeny meets biology. *Trends Parasitol.* 35(5), 356-368.
24
25 558 <https://doi.org/10.1016/j.pt.2019.01.007>.
26
27 559 Jefferies R, Down J, McInnes L, Ryan U, Robertson H, Jakob-Hoff R, Irwin P. 2008, Molecular
28
29 560 characterization of *Babesia kiviensis* from the brown kiwi (*Apteryx mantelli*). *J. Parasitol.*
30
31 561 94(2), 557-60. <https://doi.org/10.1645/GE-1344.1>.
32
33 562 Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular evolutionary
34
35 563 genetics analysis across computing platforms. *Mol. Biol. Evol.* 35:1547-1549.
36
37 564 <https://doi.org/10.1093/molbev/msy096>.
38
39 565
40
41 566 Malandrin, L., Jouglin, M., Sun, Y., Brisseau, N., Chauvin, A., 2010. Redescription of *Babesia*
42
43 567 *capreoli* (Enigk and Friedhoff, 1962) from roe deer (*Capreolus capreolus*): isolation,
44
45 568 cultivation, host specificity, molecular characterisation and differentiation from *Babesia*
46
47 569 *divergens*. *Int. J. Parasitol.* 40(3), 277-84. <https://doi.org/10.1016/j.ijpara.2009.08.008>.
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

570

1
2 571 Martinez, J., Vasquez, R.A., Venegas, C., Merino, S., 2015. Molecular characterisation of
3
4 572 haemoparasites in forest birds from Robinson Crusoe Island: Is the Austral Thrush a potential
5
6
7 573 threat to endemic birds? *Bird Conserv. Int.* 25(2), 139 – 152.
8
9
10 574 <https://doi.org/10.1017/S0959270914000227>

11 575

12
13
14 576 McCoy, K.D, Dietrich, M., Jaeger A., Wilkinson D.A., Bastien M., Lagadec E., Boulinier, T.,
15
16
17 577 Pascalis, H., Tortosa, P., Le Corre M., 2016. The role of seabirds of the Iles Eparses as
18
19 578 reservoirs and disseminators of parasites and pathogens. *Acta oecologica* 72, 98-109.
20
21
22 579 <https://doi.org/10.1016/j.actao.2015.12.013>

23 580

24
25
26 581 Merino, S., 1998. *Babesia bennetti* n. sp. from the yellow-legged gull (*Larus cachinnans*, Aves,
27
28
29 582 Laridae) on Benidorm island, Mediterranean sea. *J. Parasitol.* 84(2), 422-424.

30 583

31
32
33
34 584 Montero, E., Gonzalez, L.M., Chaparro, A., Benzal, J., Bertellotti, M., Masero, J.A.,
35
36 585 Colominas-Ciuró, R., Vidal, V., Barbosa, A., 2016. First record of *Babesia* sp. in Antarctic
37
38
39 586 penguins. *Ticks Tick Borne Dis.* 7, 498-501. <https://doi.org/10.1016/j.ttbdis.2016.02.006>.

40 587

41
42
43 588 Papparini, A., McInnes, L.M., Di Placido, D., Mackereth, G., Tompkins, D.M., Clough, R.,
44
45
46 589 Ryan, U.M., Irwin, P.J., 2014. Piroplasms of New Zealand seabirds. *Parasitol. Res.* 113, 4407-
47
48
49 590 4414. <https://doi.org/10.1007/s00436-014-4118-z>.

50 591

51
52
53 592 Parsons, N.J., Voogt, N.M., Schaefer, A.M., Peirce, M.A., Vanstreels, R.E.T., 2017.
54
55
56 593 Occurrence of blood parasites in seabirds admitted for rehabilitation in the Western Cape, South
57
58 594 Africa, 2001-2013. *Vet. Parasitol.* 233, 52-61. <https://doi.org/10.1016/j.vetpar.2016.12.001>.

595

1
2 596 Parsons, N.J., Gous, T.A., Cranfield, M.R., Cheng, L.I., Schultz, A., Horne E., Last, R.P.,

3
4 597 Lampen, F., Ludynia, K., Bousfield, B., Strauss, V., Peirce, M.A., Vanstreels, R.E.T., 2018.

5
6
7 598 Novel vagrant records and occurrence of vector-borne pathogens in King Penguins

8
9 599 (*Aptenodytes patagonicus*) in South Africa. Polar Biol. 41, 79-86.

10
11 600 <https://doi.org/10.1007/s00300-017-2171-7>.

12
13
14 601

15
16
17 602 Peirce, M.A., 2000. A taxonomic review of avian piroplasms of the genus *Babesia* Starcovici,

18
19 603 1893. J. Nat. Hist. 34, 317-332. <https://doi.org/10.1080/002229300299507>.

20
21
22 604

23
24 605 Peirce, M.A., 2005. A checklist of the valid avian species of *Babesia* (Apicomplexa:

25
26 606 Piroplasmorida), *Haemoproteus*, *Leucocytozoon* (Apicomplexa: Haemosporida), and

27
28 607 *Hepatozoon* (Apicomplexa: Haemogregarinidae). J. Nat. Hist. 39, 3621-3632.

29
30
31 608

32
33
34 609 Peirce, M.A., Parsons, N.J., 2012. *Babesia ugwiensis*, a new species of avian piroplasm from

35
36 610 Phalacrocoracidae in South Africa. Parasite 19, 375-379.

37
38 611 <https://doi.org/10.1051/parasite/2012194375>.

39
40
41 612

42
43 613 Quillfeldt, P., Arriero, E., Martínez, J., Masello, J. F., & Merino, S. (2011). Prevalence of

44
45 614 blood parasites in seabirds-a review. Front. Zool., 8, 1-11. <https://doi.org/10.1186/1742->

46
47
48 615 9994-8-26.

49
50
51 616

52
53 617 Quillfeldt, P., Martínez, J., Bugoni, L., Mancini, P.L., Merino, S., 2014. Blood parasites in

54
55 618 noddies and boobies from Brazilian offshore islands - differences between species and

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

619 influence of nesting habitat. Parasitol 141, 399-410.
620 <https://doi.org/10.1017/S0031182013001649>.
621
622 Rataud, A., Dupraz, M., Toty, C., Blanchon, T., Vittecoq, M., Choquet, R., McCoy, K.D., 2020.
623 Evaluating functional dispersal in a nest ectoparasite and its eco-epidemiological implications.
624 Front. Vet. Sci. 7, 570157. <https://doi.org/10.3389/fvets.2020.570157>.
625
626 Schnittger, L., Yin, H., Gubbels, M.J., Beyer, D., Niemann, S., Jongejan, F., Ahmed, J.S., 2003.
627 Phylogeny of sheep and goat *Theileria* and *Babesia* parasites. Parasitol Res. 91(5), 398-406.
628 <https://doi.org/10.1007/s00436-003-0979-2>.
629
630 Schnittger, L, Rodriguez, AE, Florin-Christensen, M, Morrison, D.A., 2012. *Babesia*: a world
631 emerging. Infect. Genet. Evol. 12:1788-809. <https://doi.org/10.1016/j.meegid.2012.07.004>.
632
633 Tamura, K., Nei, M.,1993. Estimation of the number of nucleotide substitutions in the control
634 region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* 10, 512-526.
635
636 Tavaré, S.,1986. Some probabilistic and statistical problems in the analysis of DNA
637 sequences in lectures on mathematics in the life sciences. Am. Math. Soc. 17, 57–86.
638
639 Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The
640 CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by
641 quality analysis tools. Nucleic Acids Res. 25, 4876-4882.
642

643 Vanstreels, R.E.T., Woehler, E.J., Ruoppolo, V., Vertigan, P., Carlile, N., Priddel, D., Finger,
1
2 644 A., Dann, P., Herrin, K.V., Thompson, P., Ferreira Junior, F.C., Braga, É.M., Hurtado, R.,
3
4 645 Epiphanio, S., Catão-Dias, J.L., 2015. Epidemiology and molecular phylogeny of *Babesia* sp.
5
6 646 in little penguins *Eudyptula minor* in Australia. *Int. J. Parasitol. Parasites Wildl.* 4, 198-205.
7
8
9 647 <https://doi.org/10.1016/j.ijppaw.2015.03.002>.
10
11
12 648
13
14 649 Work, T.M., Rameyer, R.A., 1997. Description and epizootiology of *Babesia poelea* n. sp. in
15
16 650 brown boobies (*Sula leucogaster* (Boddaert)) on sand island, Johnston Atoll, central Pacific. *J.*
17
18 651 *Parasitol.* 83, 734-738.
19
20
21 652
22
23 653 Yabsley, M.J., Work, T.M., Rameyer, R.A., 2006. Molecular phylogeny of *Babesia poelea*
24
25 654 from brown boobies (*Sula leucogaster*) from Johnston Atoll, Central Pacific. *J. Parasitol.* 92,
26
27 655 423-425.
28
29
30 656
31
32 657 Yabsley, M.J., Greiner, E., Tseng, F.S., Garner, M.M., Nordhausen, R.W., Ziccardi, M.H., et
33
34 658 al., 2009. Description of novel *Babesia* species and associated lesions from common murre
35
36 659 (*Uria aalge*) from California. *J. Parasitol.* 95, 1183-1188.
37
38
39 660
40
41 661 Yabsley, M.J., Vanstreels, R.E.T., Shock, B.C., Purdee, M., Horne, E.C., Peirce, M.A., Parsons,
42
43 662 N.J., 2017. Molecular characterization of *Babesia peircei* and *Babesia ugwidensis* provides
44
45 663 insight into the evolution and host specificity of avian piroplasmids. *Int. J. Parasitol. Parasites*
46
47 664 *Wildl.* 6(3):257-264. <https://doi.org/10.1016/j.ijppaw.2017.08.006>.
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

665 **List and legends of tables and figures**

1
2 666 **Figure 1:** Morphology of *Babesia* sp. YLG on whole blood MGG stained smears. Different
3
4
5 667 trophozoite sizes and locations within the red blood cells are shown in frames A, B and G.
6
7 668 Presence of two trophozoites in one RBC is visible in frames B and D. Localization of
8
9
10 669 chromatin as single or multiple dots is shown in frames A, C, E, G. Schizonts with different
11
12 670 shapes are shown in frames A, C, E and H (udder shape). Dividing parasites with central
13
14
15 671 remainings of cytoplasm and distal chromatin dots are depicted in frames C and G. Merozoites
16
17 672 after division are shown in frames C, D and F. Bar: 10 μ m.

18
19 673 **Figure 2.** Polymorphism of *Babesia* 18S rRNA gene sequences from the Percei group. (A)
20
21
22 674 GenBank accession numbers correspond to *Babesia* sp. YLG (MZ541058), *B. poelea*
23
24 675 (DQ200887), *Babesia* sp. 211 (KC754965), *B. peircei* (MF288025), *B. ugwidensis*
25
26 676 (MF288027), *B. uriae* (FJ717705), *B. poelea*-like I (MF162302), *B. poelea*-like II (MF162305)
27
28
29 677 and *Babesia* sp. RETV-2015 (KP144322). Sequences were compared to the 18S rRNA gene
30
31 678 sequence of *Babesia* sp. YLG. Positions correspond to the longest 18S rRNA gene sequence,
32
33
34 679 DQ200887. Nucleotides and insertions/deletions (-) are mentioned when a difference was
35
36 680 observed. Polymorphisms specific to *Babesia* sp. YLG 18S rRNA gene are colored in red. An
37
38
39 681 absence of symbols indicates identity with *Babesia* sp. YLG from the present study. (B)
40
41 682 Schematic localization of polymorphisms along the 9 aligned sequences. Each vertical bar
42
43
44 683 corresponds to a polymorphism, and those specific to *Babesia* sp. YLG 18S rRNA gene are
45
46 684 colored in red and indicated with a red arrow.

47
48
49
50 685 **Figure 3:** Maximum likelihood phylogenetic tree of *Piroplasmida* species based on partial 18S
51
52 686 rRNA gene sequences (1291 bp). Branch support/bootstrap values above 60% are indicated at
53
54
55 687 each corresponding node. Yellow-legged gull derived sequence obtained in the present study is
56
57 688 highlighted in red, and the avian *Babesia* species are colored in blue. Branch lengths are drawn
58
59
60 689 proportionally to evolutionary distance.

690 **Figure 4:** Maximum likelihood phylogenetic tree of *Piroplasmida* species based on partial *cox1*
1
2 691 gene sequences (951 bp). Branch support/bootstrap values (above 60% only shown) are
3
4 692 indicated by each node. Yellow-legged gull sequences obtained in this present study are
5
6 693 emphasized in red, and the other avian *Babesia* species, *Babesia ardeae* is colored in blue. Branch
7
8
9 694 lengths are drawn proportionally to evolutionary distance.

10
11
12 695 **Supplementary data**

13
14
15
16 696 Supplementary data 1. Location of primers used to amplify the 18S rRNA gene. 18S Bp primers
17
18 697 (colored green) were designed to specifically amplify the 18S rRNA gene of Peircei group
19
20
21 698 *Babesia* infecting marine birds.

22
23
24
25 699 Supplementary data 2. Location of primers used to amplify the *cox1* gene of *Babesia* sp. YLG.
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

700 **Table 1:** Primers used to amplify the 18S rRNA and *cox1* genes of *Babesia* sp. GF2/GR2 amplify
 701 the *Babesia / Theileria* sl. 18S rRNA gene. Primers 18SBp have been designed to specifically
 702 amplify the 18S rRNA gene of Peircei group *Babesia* and primers COX1Bp have been designed
 703 to specifically amplify the *cox1* gene of these same *Babesia* (refer to supplementary data for
 704 the alignments used to select primers).

Gene	Organism	Primer name	Primer sequence (5'-3')	Tm (°C)	Amplicon length (bp)	Reference
18S rRNA	Apicomplexan	CRYPTOF	AACCTGGTTGATCCTGCCAGTAGTCAT	63	1728	[1]
		CRYPTOR	TGATCCTTCTGCAGGTTACACCTA			
	<i>Babesia / Theileria</i> sensu lato (sl)	BAB-GF2	GTCTTGTAATTGGAATGATGG	61	560	[2]
		BAB-GR2	CCAAAGACTTTGATTTCTCT			
<i>Babesia</i> of Peircei group	18SBp_fw	CTTGAACTTCGGTTCATGGTG	60	1529	this study	
	18SBp_rev	CTAGACGTTTCTAACGAATCAG				
<i>cox1</i>	<i>Babesia</i> of Peircei group	COX1Bp_fw	GTGGWTTAAAAATAATMACWATGG	54	1220	this study
		COX1Bp_rev	CCCATAAATCATCTGGATAATC			

705 [1] Malandrin *et al.*,2010 [2] Herwaldt *et al.*,2003.

Table 2: Available avian *Babesia* 18S rRNA sequences, GenBank references, lengths, avian hosts, countries of origin and associated publications.

<i>Babesia</i> group	GenBank accession number	Length (bp)	<i>Babesia</i> species	Host	Location	Reference	
						of original <i>Babesia</i> description	of cited sequences
Peircei (n=24)	MZ541058	1485	<i>Babesia</i> sp. YLG	<i>Larus michahellis</i>	South of France	This study	This study
	DQ200887	1764	<i>Babesia poelea</i>	<i>Sula leucogaster</i>	Central Pacific	Work and Rameyer, 1997	Yabsley et al., 2006
	MF288025	1642	<i>Babesia peircei</i>	<i>Spheniscus demersus</i>	South Africa	Earlé et al., 1993	Yabsley et al., 2017
	MF288026	1642		(n=2)	(n=2)		(n=2)
	FJ717705	1706	<i>Babesia uriae</i>	<i>Uria aalge</i>	California	Yabsley et al., 2009	Yabsley et al., 2009
	MF288027	1644	<i>Babesia ugwiidiensis</i>	<i>Phalacrocorax capensis</i>	South Africa	Peirce and Parsons, 2012	Yabsley et al., 2017
	MF288028	1644		(n=2)	(n=5)		(n=5)
	MF288029	1644		<i>Phalacrocorax neglectus</i>			
	MF288030	781		<i>Phalacrocorax capensis</i>			
	MF288031	781		(n=2)			
	KC754965	1450	<i>Babesia</i> sp. 211	<i>Sula leucogaster</i>	Brazil	Quillfeldt et al., 2014	Quillfeldt et al., 2014
				<i>Sula dactylatra</i>			
	MF162301	1441	<i>B. poelea</i> -like I	<i>Chroicocephalus scopulinus</i>	New Zealand		Paparini et al., 2014
	MF162302	1451		<i>Morus serrator</i>	(n=9)		(n=9)
	MF162303	1462		<i>Chroicocephalus scopulinus</i>			
	MF162304	1461		<i>Chroicocephalus scopulinus</i>			
	MF162308	836		<i>Chroicocephalus scopulinus</i>			
	MF162309	830		<i>Sterna striata</i>			
	MF162305	1458	<i>B. poelea</i> -like II	<i>Morus serrator</i>			
	MF162306	1430		<i>Morus serrator</i>			
	MF162307	809		<i>Morus serrator</i>			
	KP144322	1538	<i>Babesia</i> sp. RETV-2015	<i>Eudyptula minor</i>	Australia	Vanstreels et al., 2015	Vanstreels et al., 2015
	KP144323	1529		(n=2)	(n=2)		
	KY436057	1786	<i>Babesia ardeae</i>	<i>Ardea cinerea</i>	Singapore	Toumanoff, 1940	Chavatte et al., 2017
KT800053	274	<i>Babesia</i> sp. EM-2016	<i>Pygoscelis antarctica</i>	Antartica	Montero et al., 2016	Montero et al., 2016	
Bennetti	DQ402155	1648	<i>Babesia bennetti</i>	<i>Larus cachinnans</i>	Spain	Merino et al., 1998	Criado et al., 2006
Kiwiensis (n=6)	EF551335	1509	<i>Babesia kiwiensis</i>	<i>Apteryx mantelli</i>	New Zealand	Peirce et al., 2003	Jefferies et al., 2008
	MF162310	1246	<i>B. kiwiensis</i> -like	<i>Sterna striata</i>	New Zealand		Paparini et al., 2014
	MF162311	1386		<i>Morus serrator</i>	(n=4)		
	MF162312	1373		<i>Chroicocephalus scopulinus</i>			
	MF162313	611		<i>Morus serrator</i>			
	JX984667	1350	<i>Babesia</i> sp. JFB1	<i>Turdus falklandii</i>	Chile		Martinez et al., 2015

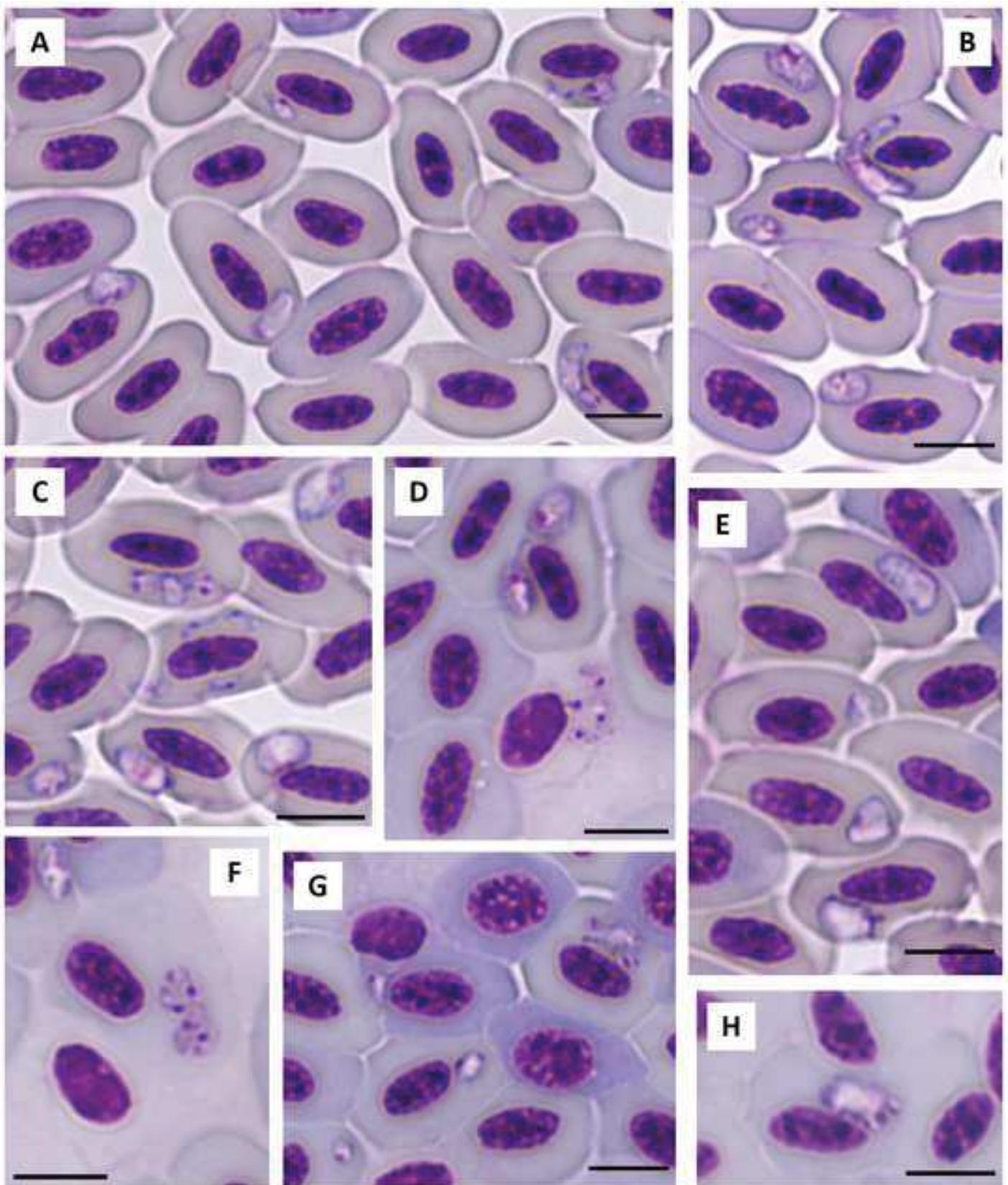
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Table 3. Parasitemia and piroplasm characterization in yellow legged gull chicks in 2019. Primers' specificity: GF2/GR2 for *Babesia / Theileria sl* 18S rRNA gene, 18SBp for 18S rRNA gene of *Babesia* of Peircei group, and COX1Bp for *cox1* gene of *Babesia* of Peircei group. Amplified *cox1* gene sequences lengths are all 1172 bp.

Gull number	Nest number	Estimated parasitemia	Amplification of 18S rRNA gene		Amplification of <i>cox1</i> gene		
			GF2/GR2 primers	Amplification (length)	COX1Bp primers	<i>cox1</i> variant	GenBank accession number
EA673739	200	> 10 %	+	-	nd		
EA673744	220	> 0,1 %	+	+(1468 bp)	nd		
EA673745	163:C	0,5 – 1 %	+	+(1472 bp)	+	b / e	MZ558163 / MZ558166
EA673746	311:C	1 %	+	+(1473 bp)	+	d	MZ558165
EA673747	205bis	0	-	-	nd		
EA673828	166:C	> 10 %	+	+(1466 bp)	nd		
EA673852	147:C	5 – 10 %	+	+(1485 bp)	+	a	MZ558162
EA673853	16	0	+	+(1477 bp)	nd		
EA673854	29	2 – 5 %	+	+(1485 bp)	nd		
EA673863	259:C	> 10 %	+	+(1485 bp)	+	a	MZ558162
EA673873	270:C	> 10 %	+	+(1485 bp)	+	a	MZ558162
EA673901	237	1 %	+	+(1461 bp)	nd		
EA673911	179:C	10 – 20 %	+	+(1485 bp)	+	a / b	MZ558162 / MZ558163
EA673919	161:C	10 %	+	+(1485 bp)	+	c	MZ558164
EA673935	493:C	0	-	-	nd		
EA677402	316:C	10 %	+	+(1474 bp)	nd		
EA677418	327:C	15 – 20 %	+	+(1464 bp)	nd		
EA677438	394C	1 %	+	+(1485 bp)	+	b	MZ558163
EA677439	372:C	10 %	+	+(1485 bp)	+	a / f	MZ558162 / MZ558167
EA677440	386:C	0	-	-	nd		

1
2 **Table 4:** SNPs descriptions for the six *cox1* variants detected in *Babesia* sp. YLG, and the frequency of each
3
4
5 variant from the nine blood sampled chicks.
6
7
8
9
10

<i>cox1</i> variant	89	107	860	917	1123	Variant frequency
a	T	G	G	C	T	5/12
b	T	G	G	T	T	3/12
c	T	A	G	C	T	1/12
d	C	G	A	C	T	1/12
e	T	A	G	T	T	1/12
f	T	G	G	C	G	1/12



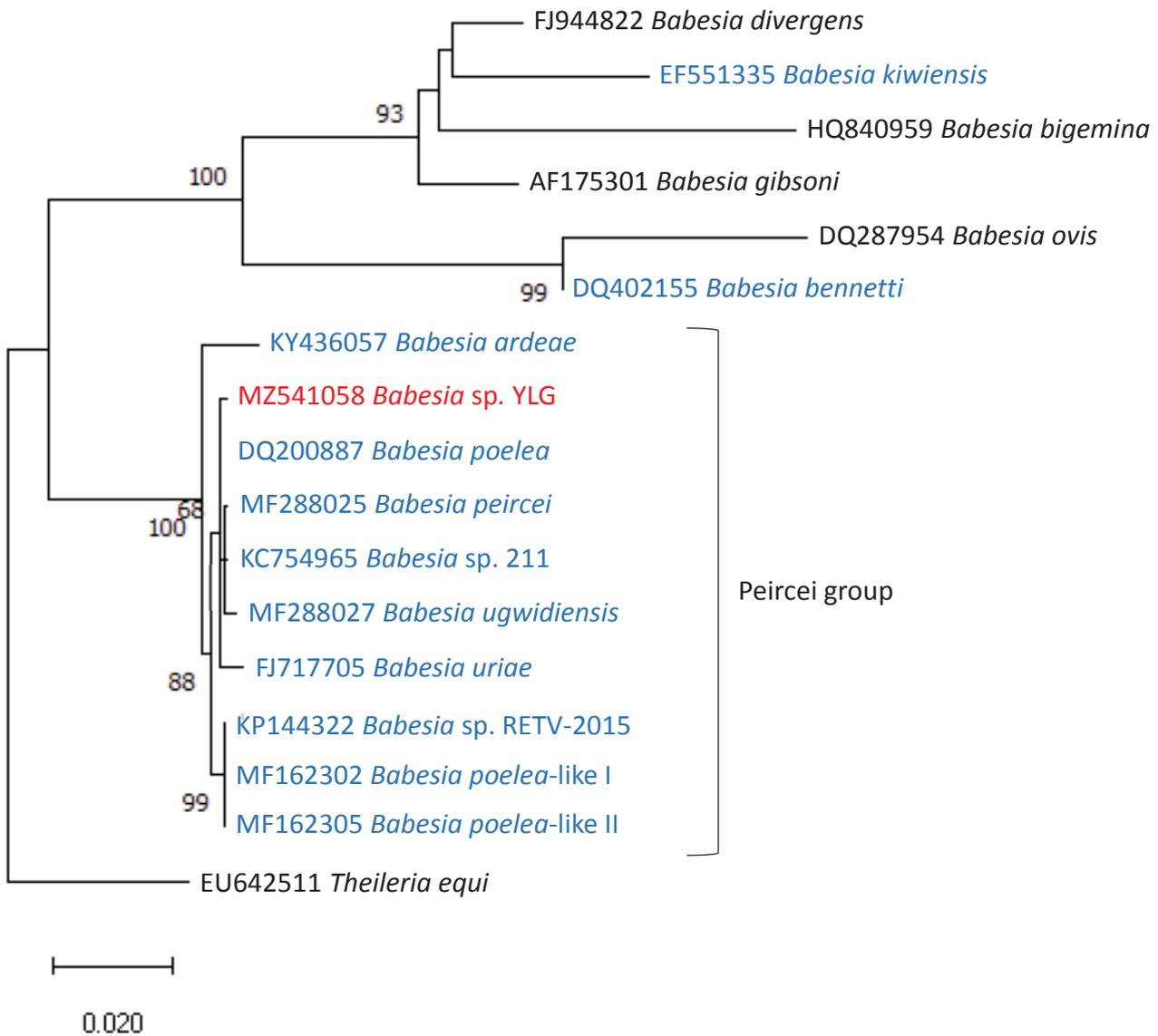


Figure 3: Maximum likelihood phylogenetic tree of *Piroplasmida* species based on partial 18S rRNA gene sequences (1291 bp). Branch support/bootstrap values above 60% are indicated at each corresponding node. Yellow-legged gull derived sequence obtained in the present study is highlighted in red, and the avian *Babesia* species are colored in blue. Scale bar indicates nucleotide substitution rate per site.

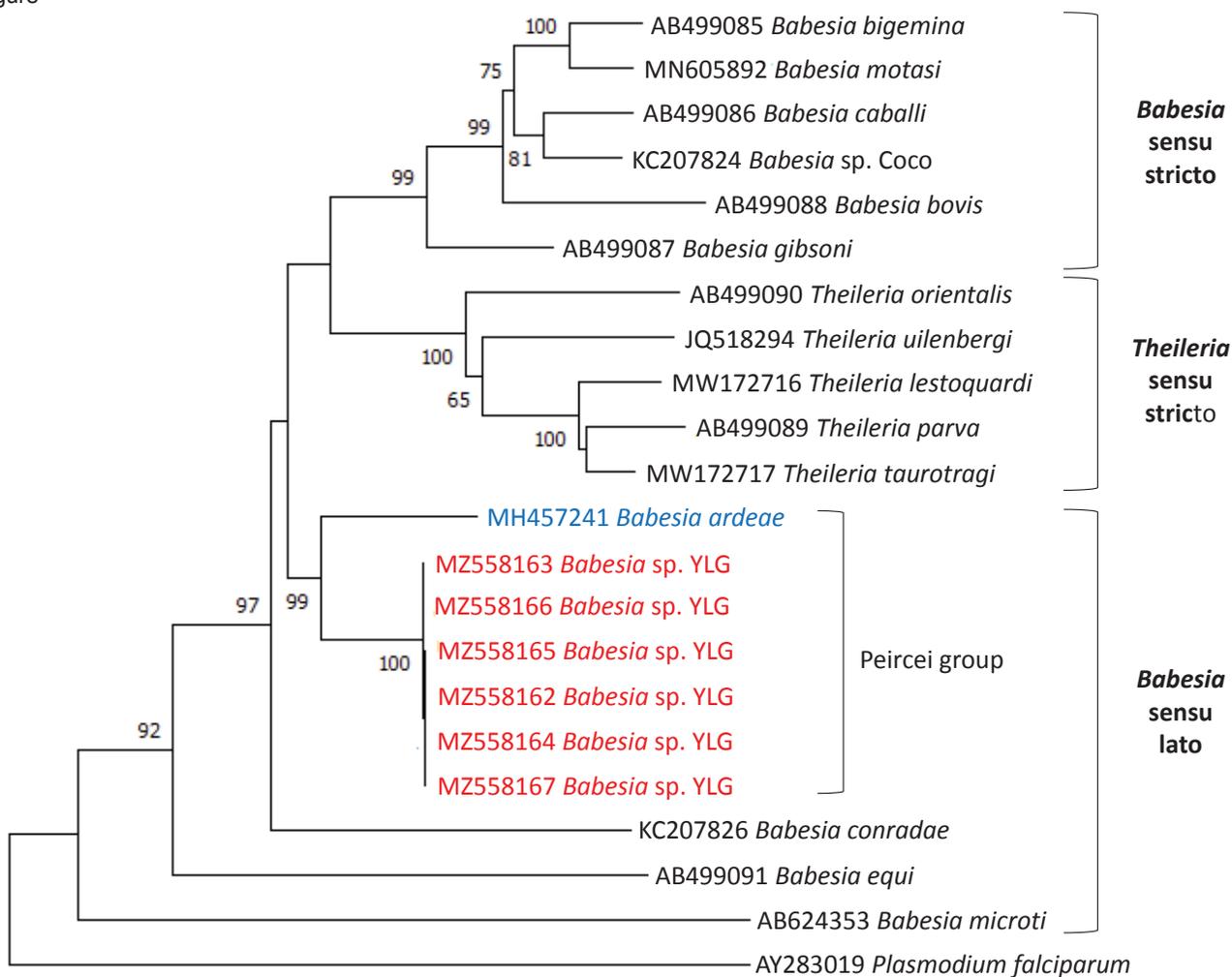


Figure 4: Maximum likelihood phylogenetic tree of *Piroplasmida* species based on partial *cox1* gene sequences (951 bp). Branch support/bootstrap values (above 60% only shown) are indicated by each node. Yellow-legged gull sequences obtained in this present study are emphasized in red, and the other avian *Babesia* species, *Babesia ardeae* is colored in blue. Scale bar indicates nucleotide substitution rate per site.

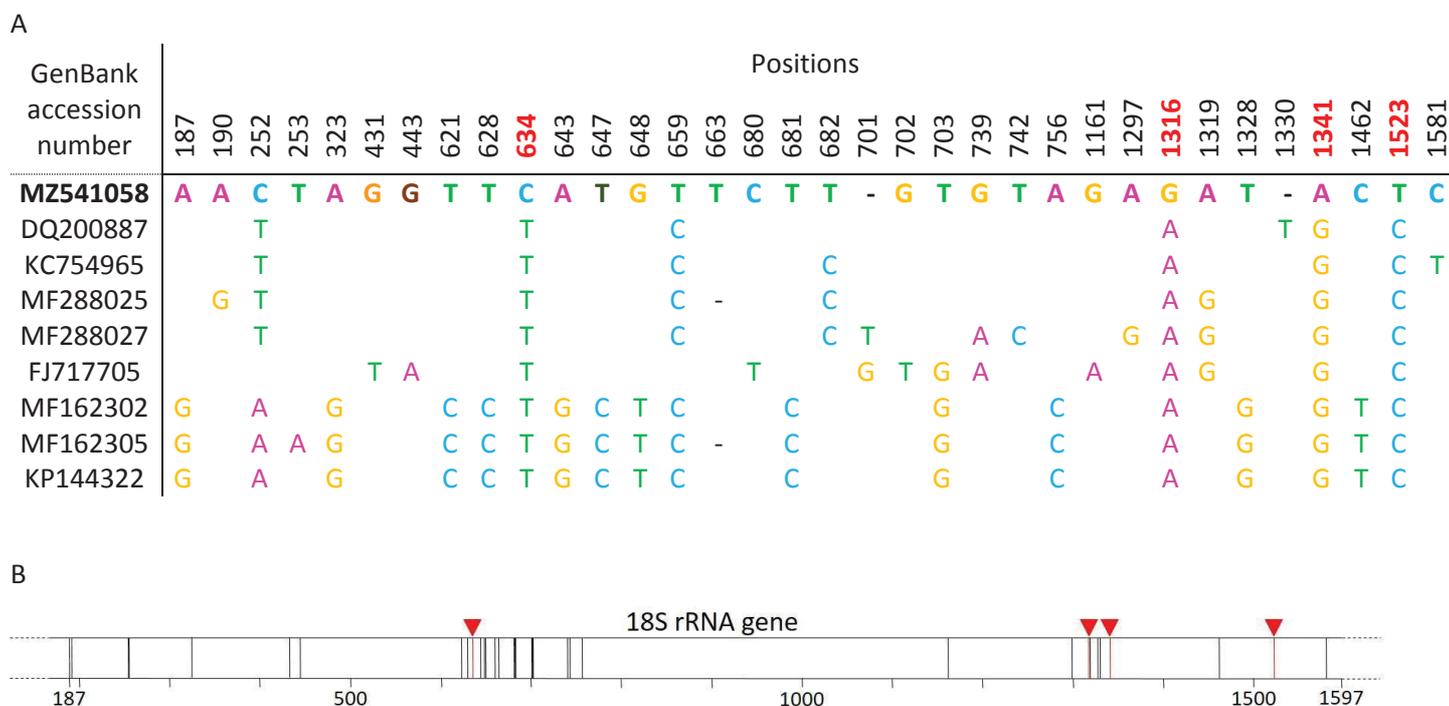


Figure 2. Polymorphism of *Babesia* 18S rRNA gene sequences from the Percei group. (A) GenBank accession numbers correspond to *Babesia* sp. YLG (MZ541058), *B. poelea* (DQ200887), *Babesia* sp. 211 (KC754965), *B. peircei* (MF288025), *B. ugwidensis* (MF288027), *B. uriae* (FJ717705), *B. poelea*-like I (MF162302), *B. poelea*-like II (MF162305) and *Babesia* sp. RETV-2015 (KP144322). Sequences were compared to the 18S rRNA gene sequence of *Babesia* sp. YLG. Positions correspond to the longest 18S rRNA gene sequence, DQ200887. Nucleotides and insertions/deletions (-) are mentioned when a difference was observed. Polymorphisms specific to *Babesia* sp. YLG 18S rRNA gene are colored in red. An absence of symbols indicates identity with *Babesia* sp. YLG from the present study. (B) Schematic localization of polymorphisms along the 9 aligned sequences. Each vertical bar corresponds to a polymorphism, and those specific to *Babesia* sp. YLG 18S rRNA gene are colored in red and indicated with a red arrow.

Acknowledgements:

We thank all the people that assisted with field sampling: Marie Buysse, Elodie Conte, Thomas Blanchon, Maxime Duhaydon, and Nathalie de la Cotte for blood smears mounting.

Funding:

Funding for this study was provided by the UMR BIOEPAR (TiBoDi team), the ANR grant EcoDIS (ANR-20-CE34-0002) and by an exploratory research grant DISTIC from the Labex CeMEB (Centre Méditerranéen de l'Environnement et de la Biodiversité) with the support an ANR "Investissements d'avenir" program (ANR-10-LABX-04-01).

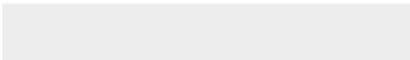
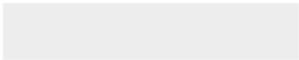
Conflict of interest: none.

Authors contribution:

Claire Bonsergent: Investigation, Formal analysis, Methodology, Visualization. **Marion Vittecoq** Resources, Supervision, Investigation, Reviewing and Editing. **Carole Leray, Louisiane Burkart** Resources. **Karen McCoy:** Resources, Conceptualization, Funding acquisition, Reviewing and Editing. **Laurence Malandrin:** Funding acquisition, Conceptualization, Supervision, Writing-Original draft preparation.



Click here to access/download
Supplementary Material
Art43-SuppData1.docx





Click here to access/download
Supplementary Material
Art43-SuppData2.docx

