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1 **Detection of emerging tick-borne disease agents in the Alpes-**
2 **Maritimes region, Southeastern France**

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15 **Keywords: Ticks; Lyme; *Ixodes ricinus*; *Borrelia*; tick-borne diseases; MALDI-TOF;**

16 **Alpes-Maritimes; France**

17 **Abstract**

18 Lyme **borreliosis** is a zoonotic tick-borne infection representing the most frequent vector-
19 borne disease in the northern hemisphere. The **Mediterranean** rim is generally described as
20 unsuitable for the **European** vector, *Ixodes ricinus*. We conducted an epidemiological study to
21 assess whether *I. ricinus* was present and study its infection status for tick-borne bacteria.
22 Ticks originating from southeastern France were obtained from flagging sampling and
23 removed from animals and tick-bitten patients. Species level identification used
24 morphological keys and MALDI-TOF MS. Quantitative PCR and sequencing assays were
25 used to detect and identify tick-associated bacteria (*Borrelia*, *Rickettsia*, Anaplasmataceae,
26 *Bartonella*, *Coxiella burnetii*) in each specimen. A total of 1232 ticks were collected in
27 several localities. Among these, 863 were identified as *I. ricinus* (70%). Bacterial screening
28 allowed identification of Lyme group *Borrelia* among *I. ricinus* ticks originating from various
29 regional areas. Other emerging tick-borne pathogens like *Borrelia miyamotoi* and *Rickettsia*
30 species were also detected. The Alpes-Maritimes region, part of the French Riviera, harbours
31 *I. ricinus* ticks infected with Lyme group *Borrelia* and several other tick-borne bacterial
32 agents. Clinicians and outdoor activity participants should be aware of the local Lyme
33 **borreliosis** transmission risk.

34 1. Introduction

35 The emergence of tick-borne diseases (TBDs) in human has been well illustrated over 40
36 years ago by Lyme **borreliosis**; a zoonotic infection caused by bacteria of the genus *Borrelia*,
37 transmitted by hard ticks of the genus *Ixodes* (Parola and Raoult, 2001). Lyme **borreliosis**
38 cases are diagnosed throughout the northern hemisphere, including North America, Asia,
39 Russia and also Europe (Madison-Antenucci et al., 2020). In Europe, the number of reported
40 cases has significantly increased in the last years (Sykes and Makiello, 2017;
41 Vandekerckhove et al., 2019).

42 The main vector of Lyme **borreliosis** in Western Europe is *Ixodes ricinus* (Madison-Antenucci
43 et al., 2020). For years, it has been thought that *I. ricinus* was absent around the French
44 Mediterranean basin, which is often described as too dry (Boulanger et al., 2019; Boulanger et
45 al., 2018; Figoni et al., 2019; Marchant et al., 2017; Pérez-Eid, 2007). In 1975, surveying of
46 forests of the Alpes-Maritimes region, south of France, could not retrieve any specimens
47 (Gilot et al., 1975). The European Center for Disease Control (ECDC) states that no
48 information is available concerning the presence of this species in the Alpes-Maritimes region
49 (“*Ixodes ricinus* - current known distribution,” 2020). Therefore, clinicians consider the
50 absence of Lyme **borreliosis** transmission risk there. However, specialized literature states the
51 observation of *I. ricinus* in southeastern France (Estrada-Peña et al., 2018). In addition,
52 apparently autochthonous emerging Lyme **borreliosis** cases have been reported there (Chirio
53 et al., 2016).

54 Entomological investigations are one of the means that enable comprehensive study of vector-
55 borne diseases (VBDs) (Laroche et al., 2017a). The aim of this work was to check for the
56 presence of *I. ricinus* in the Alpes-Maritimes, and to detect bacteria in **collected** ticks.

57 Innovative tools including Matrix-Assisted Laser Desorption-Ionization/Time Of Flight Mass

58 Spectrometry (MALDI-TOF MS) were used for species identification (Sevestre et al., 2021;
59 Yssouf et al., 2016, 2013).

60 **2. Materials and Methods**

61 2.1 Ticks

62 Specimens were collected between June 2017 and June 2020 in the Alpes-Maritimes region,
63 by flagging method (Parola and Raoult, 2001). Choice of the sites was notably guided by
64 local entomological expertise and occurrence of suspected Lyme **borreliosis** cases in these
65 areas (**Figure 3**). All samples obtained from flagging were collected right next to trekking and
66 hunting paths and routes. Some specimens were also provided by hunters who removed them
67 from hunted animals. Tick-bitten patients presenting to outpatient infectious diseases
68 department of the University Hospital of Nice also provided specimens.

69 Each tick was morphologically identified at the species **level** using identification keys
70 (Estrada-Peña et al., 2018; Pérez-Eid, 2007), using a microscope at a magnification of x56
71 (Diarra et al., 2017), then stored at -20°C until further analyses.

72 Using a sterile surgical blade, for adult stages four legs were removed. In nymphal stages 6
73 up to 8 legs were removed for MALDI-TOF MS analyses. The rest of the tick's body was
74 longitudinally cut in two equal parts, one half being used for DNA extraction and the other
75 half kept as a backup sample at -80°C.

76 2.2 MALDI-TOF MS analyses

77 For each specimen, the dissected legs were transferred in a 2-ml Eppendorf tube with a pinch
78 of 1-millimetre diameter glass beads and 20 µL of protein extraction mix (70% formic acid
79 (v/v) and 50% acetonitrile (v/v)). Protein extraction from the legs was performed and one

80 microliter of each protein extract was spotted in quadruplicates on the MALDI target
81 plate(Diarra et al., 2017; Nebbak et al., 2016).

82 Protein mass spectra were obtained with a Microflex LT MALDI-TOF mass spectrometer
83 (Bruker Daltonics, Germany)(Kumsa et al., 2016). Protein mass profiles were visualized
84 using the Flex analysis v.3.3 software and were exported to MALDI-Biotyper v.3.0 for
85 analysis. The spectra obtained were queried against our in-house arthropod database (DB).
86 The reliability of identification was estimated using the LogScore Value (LSV) obtained from
87 the MALDI-Biotyper v.3.3, ranging from 0 to 3, and corresponding to the similarity between
88 the reference spectra and the samples' mass spectra. If the MALDI-TOF MS identification
89 was congruent with morphological identification with a LSV >1.7 the specimen's
90 identification at the species level was considered correct. In other cases, molecular
91 identification was performed. Moreover, if specimens could not undergo MALDI-TOF
92 analysis due to leg absence, sequencing was performed to confirm morphological
93 identification.

94 2.3 Molecular identification of ticks

95 The DNA from each half-tick was extracted as described (Diarra et al., 2017). When
96 morphological identification was impossible, or when MALDI-TOF MS identification was
97 impossible, or when morphological and MALDI-TOF identification were not congruent,
98 molecular identification was obtained by standard PCR amplification and sequencing of a
99 455-base pair fragment of the mitochondrial 16S RNA gene as described (Parola et al., 2011).
100 The PCR mix alone was used as negative control. DNA from *Rhipicephalus sanguineus sensu*
101 *lato* reared at the laboratory was used as positive control. The sequences obtained were
102 assembled and were secondarily blasted against GenBank's Basic Local Alignment Search
103 Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov>) as described (Diarra et al., 2017).

104 2.4 Bacterial detection

105 Bacteria were detected by quantitative PCR (qPCR) as described (Diarra et al., 2017). All
106 samples were submitted to screening for the following: *Borrelia* spp., *Rickettsia* spp.,
107 *Bartonella* spp., *Coxiella burnetii* and Anaplasmataceae. Target genes, primers and probes
108 sequences were used as previously described (Diarra et al., 2017; Klee et al., 2006). The DNA
109 from our laboratory-cultured strains of *Borrelia crocidurae*, *Rickettsia africae*, *Bartonella*
110 *quintana*, *C. burnetii* and *Ehrlichia canis* were respectively used as positive controls. DNA
111 originating from uninfected laboratory-reared *Rh. sanguineus sensu lato* ticks was used as a
112 negative control. Samples were considered positive with Ct<36 for all bacteria tested.

113 All samples positive for *Borrelia* spp. were submitted to mitochondrial 16S gene standard
114 PCR amplification and **sequences** as described (Parola et al., 2011). Samples that could not be
115 sequenced with 16S gene were submitted to Flagellin B gene (*flaB*) standard PCR and
116 **sequences** as described (Socolovschi et al., 2012). Positive samples for *Rickettsia* spp. were
117 submitted to a specific qPCR system accordingly to the tick species: *Rickettsia helvetica*
118 qPCR for *I. ricinus* (Davoust et al., 2012), and *Rickettsia slovaca* and *Rickettsia raoultii*
119 qPCR for *Dermacentor* spp. (Chisu et al., 2017). Positive samples originating from other tick
120 species were submitted to *OmpA* standard PCR and sequencing as described (Yssouf et al.,
121 2014). Samples positive for Anaplasmataceae underwent 23S gene standard PCR
122 amplification and sequencing as described (Diarra et al., 2017). Samples positive for
123 *Bartonella* spp. were sequenced using the *ftsZ* gene as described (Diarra et al., 2017).

124 2.5 Phylogenetic analysis

125 We drew phylogenetic trees **with** alignment of obtained sequences for two genes (16S rRNA
126 gene and Flagellin B gene) used in this study for *Borrelia* identification. Phylogenetic trees
127 were drawn as described (Laroche et al., 2017b).

128 3. Results

129 3.1 Ticks

130 A total of 1232 ticks was obtained **between June 2017 and June 2020 in the Alpes-Maritimes**
131 **region, at altitudes ranging from 450 m up to 1300 m**. Among those, 1069 (86.76%) were
132 **obtained** from flagging collections. Also, 107 (8.6%) specimens were retrieved from patients,
133 including 71 on consulting patients. Most patients had reported outdoor activities in the
134 eastern Alpes-Maritimes. Moreover, 56 (4%) specimens were originating from animals in the
135 same period of time (either from hunted animals, or from domestic animals). Only 20/1232
136 (2.2%) of the specimens were engorged. A majority of *I. ricinus* nymphs were obtained.
137 Stages and species level morphological identification are summarized in Table 1.

138 3.2 MALDI-TOF MS Identification

139 A total of 1211/1232 ticks were submitted to MALDI-TOF MS analysis. Species-specific
140 spectra of good quality were obtained with 1171/1211 (95%) samples (Figure 1). Specimens
141 morphologically identified as *Haemaphysalis punctata* and *Ixodes acuminatus* were not
142 identified with MALDI-TOF MS, as our DB did not contain reference spectra for these two
143 species. Further molecular identification of those specimens allowed ensuring of the species
144 level identification. We secondarily included eight reference spectra for these two species in
145 our DB. When matched against our in-house database, 1146/1171 (97.86%) of the specimens
146 were correctly identified with a LSV>1.7. In the end, for all the specimens correctly
147 identified, LSVs ranged between 1.7 – 2.795, with an average of 1.940 and a median of 1.958.
148 Regardless of the LSV threshold, the total of tick specimens properly identified at the species
149 level represents 1166/1171 (99.57%) specimens.

150 3.3 Tick molecular identification

151 Eighty-eight ticks were submitted to 16S sequencing: 6 because of extensive damage
152 hampering species level identification, 15 others which could be morphologically identified at
153 the species level but could not be submitted to MALDI-TOF because of leg absence.
154 Moreover, 40 tick specimens who provided low quality spectra were also sequenced to
155 confirm morphological identification. Twenty-five ticks were also sequenced because of
156 inconsistent MALDI-TOF MS identification. Concerning two morphologically identified
157 species that were not initially present in the DB, sequencing was performed to confirm
158 morphological identification. Molecular identification confirmed the identification for *H.*
159 *punctata*, whereas our specimen identified as *I. acuminatus* was identified as *Ixodes frontalis*
160 (Id: 99.76%, MF370647). Using specimen pictures taken during the morphological
161 identification step, new performing of morphological identification by an expert of the team
162 appeared to be consistent with the molecular identification. Among the six extensively
163 damaged specimens, two were identified as *I. ricinus*, one as *Rh. bursa*, one as *Rh.*
164 *sanguineus sensu lato*, and for the two left no sequence could be obtained. All other
165 specimens had 16S sequence identification consistent with morphological identification.

166 3.4 Detection of bacteria

167 Bacterial DNA was detected from 153/1232 ticks (12.4%), including *Borrelia* (91/1232; 7%).
168 Most *Borrelia* DNA was detected in *I. ricinus* (87/91; 95%), indicating an overall 10%
169 *Borrelia* spp. infection rate for this tick species. *Rickettsia* spp. was detected in 49/1232 (3%)
170 ticks, including 23 *Dermacentor marginatus* (of which 9 specimens were engorged), 25 *I.*
171 *ricinus* (of which 1 specimen was engorged) and 1 *H. punctata*. Anaplasmataceae spp. were
172 detected in 11/1232 (0.9%) ticks, out of which 5/11 were *I. ricinus* and 6/11 to *H. punctata*.
173 *Bartonella* spp. was detected in two (2/1232) samples. No ticks were found to be positive for

174 *C. burnetii*. Developmental stages of positive tick specimens are indicated in Table 2.

175 Exception made of 10 ticks positive for *Rickettsia* spp., none of the other tick samples in
176 which bacterial DNA was detected were engorged.

177 Co-infections were observed in 3 specimens: DNA from *Borrelia* sp., *Rickettsia* sp. and
178 Anaplasmataceae were detected in the same *I. ricinus* tick. DNA from both *Borrelia* sp. and
179 *Rickettsia* sp. were detected in two other *I. ricinus* ticks.

180 In our sample, 44/91 *Borrelia* spp. samples were identified by 16S sequencing as *Borrelia*
181 *afzelii* (n=14, identification percentage ranging from 99.02% - 100%, Top Hit accession
182 numbers : AY342030.1), *Borrelia garinii* (n=11, 99.39% - 100%, AM418453.1), *Borrelia*
183 *burgdorferi* s.s. (n=3, 99.27% - 99.85%, AY342028.1), *Borrelia valaisiana* (n=6, 99.78 -
184 100%, NR_036807.1), *Borrelia miyamotoi* (n=7, 99.77% - 99.93%, KJ847049.1) and
185 *Borrelia* sp. (n=3, 99.56% - 100%, LC170035.1). *Borrelia* species infection according to tick
186 species is summarized in Table 2.

187 Out of the 47 samples that had not been identified after 16S standard PCR, 33 could be
188 sequenced after *flaB* standard PCR, allowing identification of *B. afzelii* (n=17, 97.4% -
189 99.6%, HM802194.1) *B. garinii* (n=6, 99.02% - 100%, MN325939.1), *B. burgdorferi* s.s.
190 (n=2, 98.76% - 99.43%, MK604273.1), *B. valaisiana* (n=2, 99.35% - 99.70%), *B. miyamotoi*
191 (n=5, 99.35% - 99.70%, CP046389.1) and *Borrelia* sp. (n=1, 99.05%, KR677089.1). *Borrelia*
192 species infection accordingly to the tick species is summarized in Table 2.

193 Fourteen specimens positive for *Borrelia* spp. screening remained impossible to sequence
194 with the two target genes. Combination of the two sequencing systems results gave a total of
195 31 *I. ricinus* ticks infected with *B. afzelii* (31/863; 3.6%), 17 infected with *B. garinii* (17/863;
196 2%), 5 with *B. burgdorferi* s.s. (5/863; 0.5%), 8 with *B. valaisiana* (8/863; 0.9%), and 12 with
197 *B. miyamotoi* (12/863; 1.4%). Phylogenetic analysis of some of the sequenced *Borrelia* was in

198 accordance with molecular identification (Figure 2A and 2B). For the *Borrelia* species
199 aforementioned, all of the infected ticks were identified as *I. ricinus*. Five of the ticks tested
200 positive for *B. afzelii* originated from patients of the Nice University Hospital.

201 The 25 *Rickettsia* positive *I. ricinus* ticks were submitted to a *R. helvetica* specific qPCR,
202 which detected *R. helvetica* in 22/25 ticks. Among the 23 positive *D. marginatus*, 17/23 ticks
203 were positive with *R. slovaca* specific qPCR. The 6 others *D. marginatus* ticks were found
204 positive when submitted to a *R. raoultii* specific qPCR assay. The 3 remaining *I. ricinus* ticks
205 and the only *H. punctata* positive for *Rickettsia* sp. were submitted to *OmpA* standard PCR in
206 order to sequence, but no DNA could be detected after electrophoresis despite repeated
207 attempts.

208 For Anaplasmataceae, 23S standard PCR detected DNA in 9/11 samples, and sequencing
209 allowed identification of *Anaplasma phagocytophilum* in 3 *I. ricinus* ticks (98.10% - 98.96%,
210 KM021415.1), and *Wolbachia* sp. in 2 *I. ricinus* ticks and 4 *H. punctata* ticks (99.55% -
211 100%, KT827385.1, NR_076629.1, NR_076253.1). One of the *I. ricinus* ticks testing positive
212 for *A. phagocytophilum* originated from a Nice University Hospital patient. For the two ticks
213 positive for *Bartonella* spp., standard PCR targeting the *ftsZ* gene did not detect DNA after
214 electrophoresis. Results of molecular identification are summarized in Table 2.

215 4. Discussion

216 Our results indicate an unambiguous Lyme borreliosis transmission risk in the Alpes-
217 Maritimes region of the French Riviera (Figure 3). Indeed, this area harbours biotopes that are
218 compatible with development and proliferation of telotrophic and hygrophilic ticks such as *I.*
219 *ricinus*. We also identified several potential human pathogens in questing ticks and from ticks
220 removed from human and animals.

221 Morphological identification of ticks at the species level requires expertise and remains **time**
222 **consuming**, even for skilled entomologists (Murugaiyan and Roesler, 2017). As shown in our
223 work with the example of *I. frontalis*, misidentification may occur. Systematic sequencing of
224 the specimen **before** being added to the MALDI-TOF MS DB allows us to overcome MI
225 errors. **With this approach, identification of closely related *Ixodes* species often combine two**
226 **target genes such as the 16S and cytochrome oxidase subunit I (Boyer et al., 2019). Thus, our**
227 **molecular identification confidence could be limited by the sole use of 16S sequencing.**
228 **Nevertheless, many research papers use the 16S gene for *Ixodes* molecular identification,**
229 **including expert works for description of new *Ixodes* species (Chițimia-Dobler et al., 2016).**
230 Finally, this study confirms that MALDI-TOF MS is an accurate emerging identification tool
231 in medical entomology (Sevestre et al., 2021; Yssouf et al., 2016, 2013), requiring no
232 entomological expertise.

233 *Ixodes ricinus* was found in various spots and landscapes in a region where its presence was
234 uncertain. *Ixodes ricinus* is an hygrophilic tick, very sensitive to **desiccation**, and usually is
235 stated **to be** absent from dry landscapes which lack the humidity this tick requires in order to
236 survive (Boulanger et al., 2018). **Thus, it is unlikely that this tick could proliferate on the**
237 **urbanized coastline.** The presence of grass is important for its questing activity, and the
238 presence of a leaf-litter (as found in deciduous forests) is also important for resting **and water**
239 **replenishment** (Ehrmann et al., 2017). **During this survey, we investigated mostly deciduous**
240 **forests, mixed forests (e.g. deciduous and conifers) and also a few pastures. When flagging in**
241 **Courmes (Figure 3, label D), we surveyed pastures that contained a significant proportion of**
242 ***H. punctata*. Nevertheless, in all the surveyed biotopes, we collected a majority of *I. ricinus*.**
243 **Interestingly, these results are in accordance with what has been previously described in the**
244 **neighboring Italian Liguria region (Ceballos et al., 2014). Concerning the Alpes-Maritimes,**
245 **we hypothesize that tick populations do not depend much on the geographical location itself,**

246 but rather on the region's particular geology, as it is made of multiple valleys, which allow
247 partial exposure to sunlight and thus create suitable habitats for hygrophilic ticks. The
248 existence of hosts (e.g. cervids) that may carry ticks in other remote ecological niches could
249 be another explanation for the wide distribution of *I. ricinus* in this region. Indeed, we
250 collected *I. ricinus* specimens in altitudes ranging from 450 m up to 1300 m, where some of
251 the landscapes represent an ideal biotope for this tick, functioning as fresh and humid
252 ecological niches in a region with a Mediterranean climate, in which summer months are
253 rather hot and dry. Furthermore, our sampling did not detect any *Ixodes inopinatus* specimens,
254 even though this species has been described in Mediterranean regions (Estrada-Peña et al.,
255 2014). Indeed, neither morphological identification nor molecular identification following
256 MALDI-TOF MS identified this species in our study sample. Further samplings and study
257 should allow assessment of this species' distribution in the Alpes-Maritimes region.

258 Another crucial point for tick proliferation is the existence of suitable hosts that ensure
259 dissemination (Parola and Raoult, 2001). During the field sampling sessions, we observed
260 numerous cervids (*Capreolus capreolus*, *Cervus elaphus*), as well as indications of their
261 presence (i.e. feces and footprints). These cervids are known to be suitable hosts for *Ixodes*
262 ticks, and play a role in vector dissemination (Díaz et al., 2019). Interestingly, *C. elaphus* has
263 been largely introduced in the region during the last century for game hunting (Rolland,
264 2003). This might have played a part in dissemination of *I. ricinus* in the region, since no
265 specimens of this tick were found while surveying the Alpes-Maritimes forests in 1975
266 (GILOT et al., 1975).

267 To the best of our knowledge, the present work represents the first detection of European
268 Lyme group *Borrelia* (*B. afzelii*, *B. garinii*, *B. burgdorferi* s.s.) in questing ticks in
269 southeastern France. We observed a *Borrelia* spp. infection rate of 10.1% in *I. ricinus*,
270 regardless of the collection method and developmental stages. This infection rate is

271 comparable to the ones observed through surveillance of questing ticks in metropolitan
272 France, depending on the endemic regions surveyed (Boulanger et al., 2018; Cotté et al.,
273 2009; Rauter and Hartung, 2005). Moreover, the fact that the infected ticks were collected in
274 the immediate surroundings to trekking and hunting pathways highlights a potential hazard
275 for outdoor professionals and leisure participants. The detection of Lyme group *Borrelia* in
276 ticks **obtained** from patients practicing outdoor activities underlines the reality of this
277 transmission risk. We also observed infection with *B. valaisiana* in *I. ricinus* ticks in two of
278 the surveyed localities. Part of the *B. burgdorferi* s.l. complex, this species has been detected
279 in *I. ricinus* ticks across Europe, with a prevalence similar to Lyme group *Borrelia* in questing
280 ticks (Cotté et al., 2009). Although DNA from this species has anecdotally been detected in
281 human tissue samples (Diza et al., 2004; Godfroid et al., 2003; Saito et al., 2007), its potential
282 participation in Lyme **borreliosis** occurrence is debated (Margos et al., 2017).

283 In *I. ricinus*, we also detected for the first time in southern France *B. miyamotoi*, an emerging
284 pathogen closely related to the **relapsing** fever group *Borrelia* (Madison-Antenucci et al.,
285 2020). It may be responsible for various unspecific clinical presentations including fevers,
286 chills, but also erythema migrans and even meningoencephalitis in immunocompromised
287 **patients** (Krause et al., 2015; Madison-Antenucci et al., 2020; Wagemakers et al., 2015).

288 Interestingly, 4 *H. punctata* were found to harbour a *Borrelia* sp. previously detected from
289 *Haemaphysalis* ticks in Japan (Lee et al., 2014). Being more closely related to the **relapsing**
290 fever-group *Borrelia*, this species has since been detected within hard ticks of the genus
291 *Haemaphysalis* and *Rhipicephalus* in Asia (Japan, Malaysia, China) and also in Europe
292 (Portugal) (Furuno et al., 2017; Khoo et al., 2017; Nunes et al., 2016; Yang et al., 2018). This
293 *Borrelia* seem to infect wild animals **such as boar and deer**, inducing bacteremia (Furuno et
294 al., 2017). High levels of **spirochetemia** were recorded among **deer** in Japan, suggesting they
295 might act as reservoir hosts (Kumagai et al., 2018). We did not find any published record of

296 detection of this **relapsing** fever-like *Borrelia* in France. The prevalence of tick infection in
297 France, as well as this *Borrelia*'s zoonotic potential should be further investigated.

298 *Rickettsia* are well-known tick-borne human pathogens (Parola et al., 2013). We detected 2
299 human pathogens; *R. slovaca* and *R. raoultii* in their usually associated *Dermacentor* ticks
300 (Parola et al., 2013). For these two pathogens, clinical presentations often consist in the
301 SENLAT syndrome, also referred to as TIBOLA (Tick-Borne LymphAdenopathy) or
302 DEBONEL (Dermacentor-Borne Neck Lymphadenopathy) (Parola et al., 2013). This
303 syndrome is defined by concomitant occurrence of a scalp eschar and neck lymphadenopathy
304 following a tick bite (Angelakis et al., 2010), and is known to occur throughout Europe
305 (Gouriet et al., 2006; Parola et al., 2013, 2009). Concerning *R. helvetica*, infection is
306 described as milder, with a probably important proportion of undiagnosed cases due to the
307 mild clinical presentations, which includes headaches and fatigue (Parola et al., 2013). Some
308 cases have however presented with rashes and inoculation eschars (Parola et al., 2005).

309 *A. phagocytophilum* was detected in three *I. ricinus* ticks. This bacteria is responsible for
310 human granulocytic anaplasmosis (Madison-Antenucci et al., 2020). Even though this agent
311 has been detected in *Ixodes* ticks worldwide, human anaplasmosis remain rare and generally
312 presents with **nonspecific** symptoms (Matei et al., 2019).

313 For some of our tick samples positive for bacterial screening, no bacterial DNA **was**
314 **detected** after standard PCR. This frequent issue is attributable to the **lower** sensitivity of
315 standard PCR compared to qPCR to detect infectious agents in ticks. The existence of PCR
316 inhibitors in ticks is well described, with a particular occurrence in bloodfed ticks (Schwartz
317 et al., 1997).

318 In conclusion, we definitely demonstrated the presence of *I. ricinus* in some areas of Alpes-
319 Maritimes, in which biotopes are favourable for its development, as well as the Lyme

320 **borreliosis** and other tick-borne diseases transmission risk endemicity in the Alpes-Maritimes
 321 region. We hope that this research will increase awareness of health practitioners in this
 322 highly touristic region of the French Riviera, as well as travel medicine specialists elsewhere
 323 (13 million tourists in 2019) (Comité Régional du Tourisme Côte d’Azur France, n.d.).
 324 Outdoor activity participants in the Alpes-Maritimes should **apply** safety procedures to ensure
 325 **avoidance of tick bites** (Razai et al., 2020), **and** to check for their presence when **returning**
 326 from their outdoor activities.

327 **Tables**

328 Table 1. Description of the morphological identification and developmental stages of all the
 329 tick specimens collected in the Alpes-Maritimes region of southeastern France between June
 330 2017 and June 2020. Ticks were collected by the flagging method and also removed from
 331 human and animal hosts. **For each stage, the number of engorged tick specimens is indicated**
 332 **in parentheses**. Larval stages were not considered in the study. (*) **The specimens**
 333 **morphologically identified as *Ixodes acuminatus* were secondarily identified by sequencing as**
 334 ***Ixodes frontalis*. All the *I. frontalis* specimens were collected using the flagging method.**

Morphological Identification	Female	Male	Nymph	Total
Impossible	-	-	2 (0)	2 (0)
<i>Ixodes</i> sp.	1 (0)	-	-	1 (0)
<i>Rhipicephalus</i> sp.	2 (2)	1	-	3 (2)
<i>Dermacentor marginatus</i>	28 (12)	27 (3)	-	55 (15)
<i>Haemaphysalis punctata</i>	8 (0)	7 (0)	124 (0)	139 (0)
<i>Ixodes acuminatus</i> (*)	4 (0)	-	10 (0)	14 (0)
<i>Ixodes ricinus</i>	47 (1)	29 (0)	787 (8)	863 (9)
<i>Rhipicephalus sanguineus</i> s.l.	8 (2)	4 (0)	1 (0)	13 (2)
<i>Rhipicephalus bursa</i>	74 (0)	68 (0)	-	142 (0)
Total	172 (17)	136 (3)	924 (8)	1232 (28)

335 Table 2: Bacterial pathogens detected in collected ticks. For each bacterium species detected,
 336 the numbers of the relevant tick species are indicated. Developmental stages are indicated
 337 below positive tick specimens (A: Adult, N: Nymph). When bacterial molecular identification
 338 was possible, the number of ticks positive for a particular bacterium species is provided. The
 339 collecting method for all the infected ticks is provided to the right. All ticks were collected in
 340 the Alpes-Maritimes region of southeastern France between June 2017 and June 2020,
 341 excepted the 18 positive *D. marginatus* removed from boar killed in Canjuers, Var Region of
 342 southeastern France.

Bacteria (qPCR)	Number of positives	Tick species	Molecular identification	Flagging	Animal	Patients
<i>Borrelia</i> sp.	91/1232	<i>H. punctata</i> (4/91)	<i>Borrelia</i> sp. (4)	4	-	-
		(N:4)				
		<i>I. ricinus</i> (87/91)	<i>B. afzelii</i> (31)	26	-	5
		(A:7, N:80)	<i>B. garinii</i> (17)	17	-	-
			<i>B. burgdorferi</i> s.s. (5)	5	-	-
			<i>B. valaisiana</i> (8)	8	-	-
		<i>B. miyamotoi</i> (12)	12	-	-	
<i>Rickettsia</i> sp.	49/1232	<i>I. ricinus</i> (25)	<i>R. helvetica</i> (22)	18	-	4
		(A:2, N:23)				
		<i>D. marginatus</i> (23)	<i>R. slovaca</i> (17)	2	14	1
		(A:23)	<i>R. raoultii</i> (6)	1	4	1
		<i>H. punctata</i> (1)	-	-	-	
		(A)				
Anaplasmataceae	11/1232	<i>H. punctata</i> (6)	<i>Wolbachia</i> sp. (4)	4	-	-
		(N:6)				
		<i>I. ricinus</i> (5)	<i>A. phagocytophilum</i> (3)	2	-	1
		(A:1, N:4)	<i>Wolbachia</i> sp. (2)	1	-	1
<i>Bartonella</i> sp.	2/1232	<i>I. ricinus</i> (1)	-	-	-	-
		(A)				
		<i>R. sanguineus</i> s.l. (1)	-	-	-	-
		(A)				
<i>Coxiella burnetii</i>	0/1232	-	-	-	-	-

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355 **Conflict of Interest**

356 The authors declare that the research was conducted in the absence of any commercial or
357 financial relationships that could be construed as a potential conflict of interest.

358 **Author Contributions**

359 JS performed tick sampling, realized morphological analyses, MALDI-TOF MS, molecular
360 biology analyses and sequencing, collected the data and wrote the original draft. AZD
361 supervised and participated to MALDI-TOF MS and molecular biology analyses. HAO

362 participated to tick sampling and MALDI-TOF MS analyses. JD provided clinical samples
363 and reviewed the manuscript. PD provided tick samples, reviewed the manuscript and
364 supervised the work. PP provided reagents and automatons, reviewed the manuscript and
365 supervised the work.

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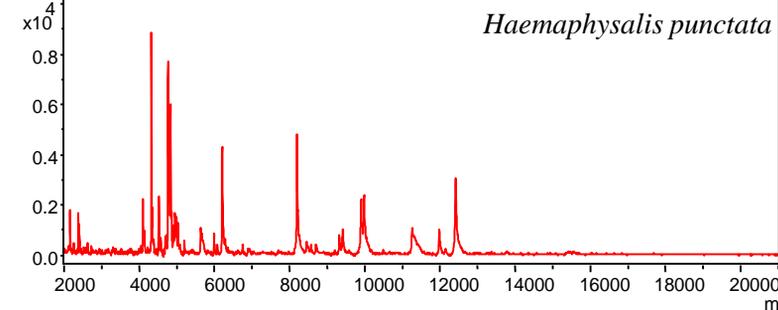
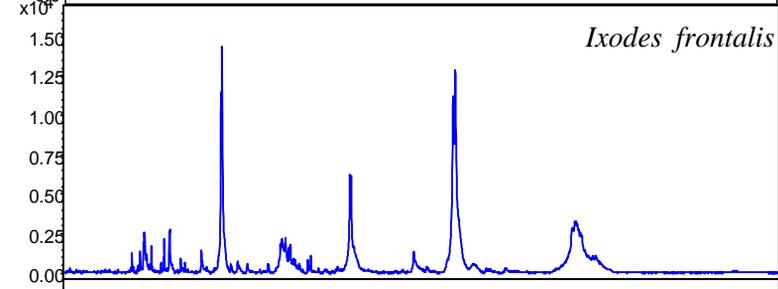
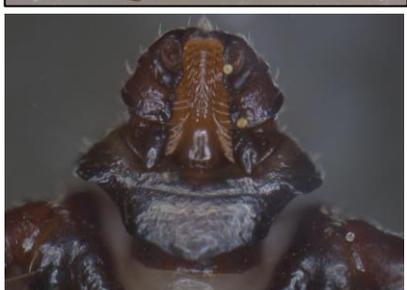
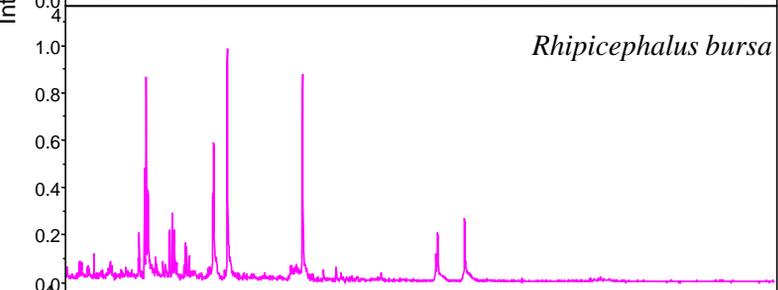
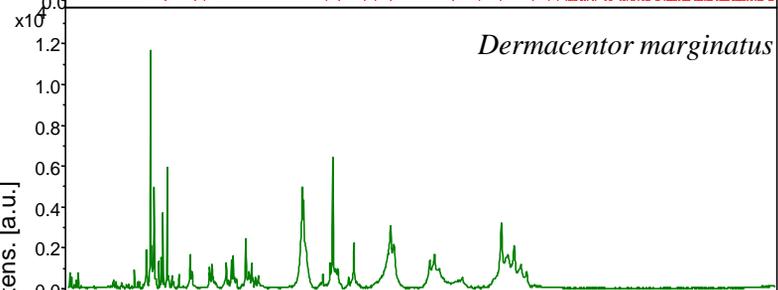
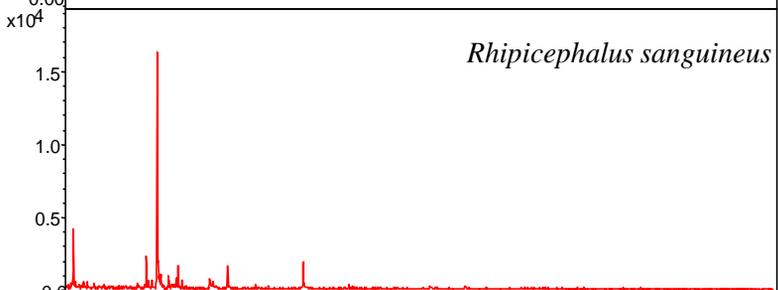
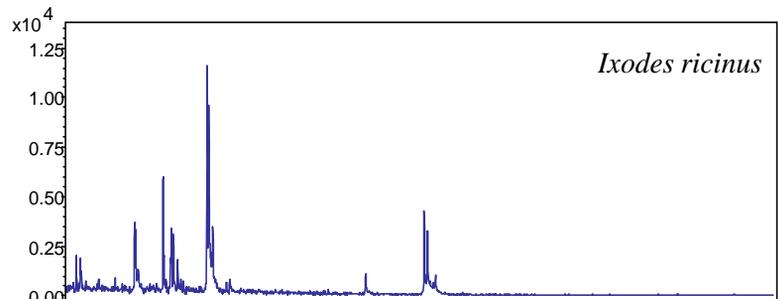
Figure Legends

Figure 1. Representation of some species-specific MALDI-TOF MS spectra obtained through MALDI-TOF MS analysis of legs of tick specimens collected between June 2017 and June 2020 in the Alpes-Maritimes region, France. Corresponding tick species appear on the right of each represented spectra.

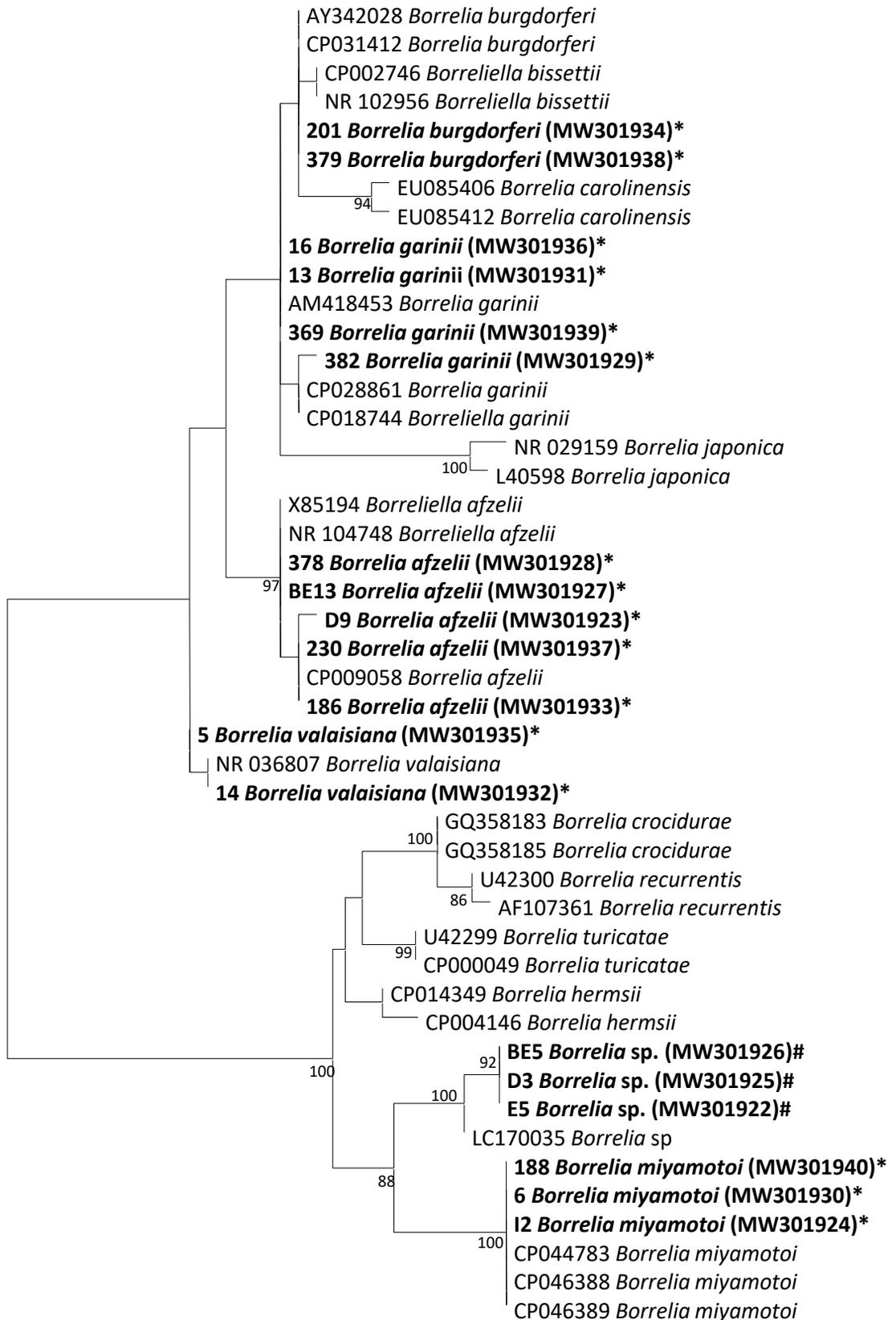
Figure 2. Phylogenetic trees showing the position of *Borrelia* amplified from *I. ricinus* (*) and *H. punctata* (#) ticks, collected in the Alpes-maritimes region of SouthEastern France between June 2017 and June 2020, (in bold) compared to other isolates of the same and different species. Sequences have been deposited in GenBank, with the corresponding accession numbers between brackets. Phylogenetic inferences were obtained with MEGA 6. Genbank accession numbers are indicated at the beginning. The 16S rRNA gene sequence (Panel A) and the Flagellin B gene sequences (Panel B) were aligned with CLUSTALW. Phylogenetic inferences were obtained using ML phylogenetic analysis with TOPALI 2.5 software (Biomathematics and Statistics Scotland, Edimburgh, UK) using the integrated ML application. Numbers at the nodes are percentages of bootstraps values obtained by repeating the analysis 100 times to generate a majority consensus tree (for these trees, only bootstraps values equal or higher than 80 were retained).

Figure 3. Map of the Alpes-Maritimes region of southeastern France showing the geographical areas in which ticks were collected by flagging method between June 2017 and June 2020 : A. Tende, B. La Brigue, C. Breil-Sur-Roya, D. Moulinet, E. Levens, F. Courmes and Bar-Sur-Loup, G. Caussols . The Roya Valley was investigated in the forests surrounding Tende (44.0889416,7.5933916) in May 2020 and Breil-sur-Roya (43.9375321,7.5139664) in March 2020. The Levenza Valley was also surveyed, in the forests surrounding La Brigue (44.0618551,7.6158933) in September 2019 and May 2020. The Bevera valley was also

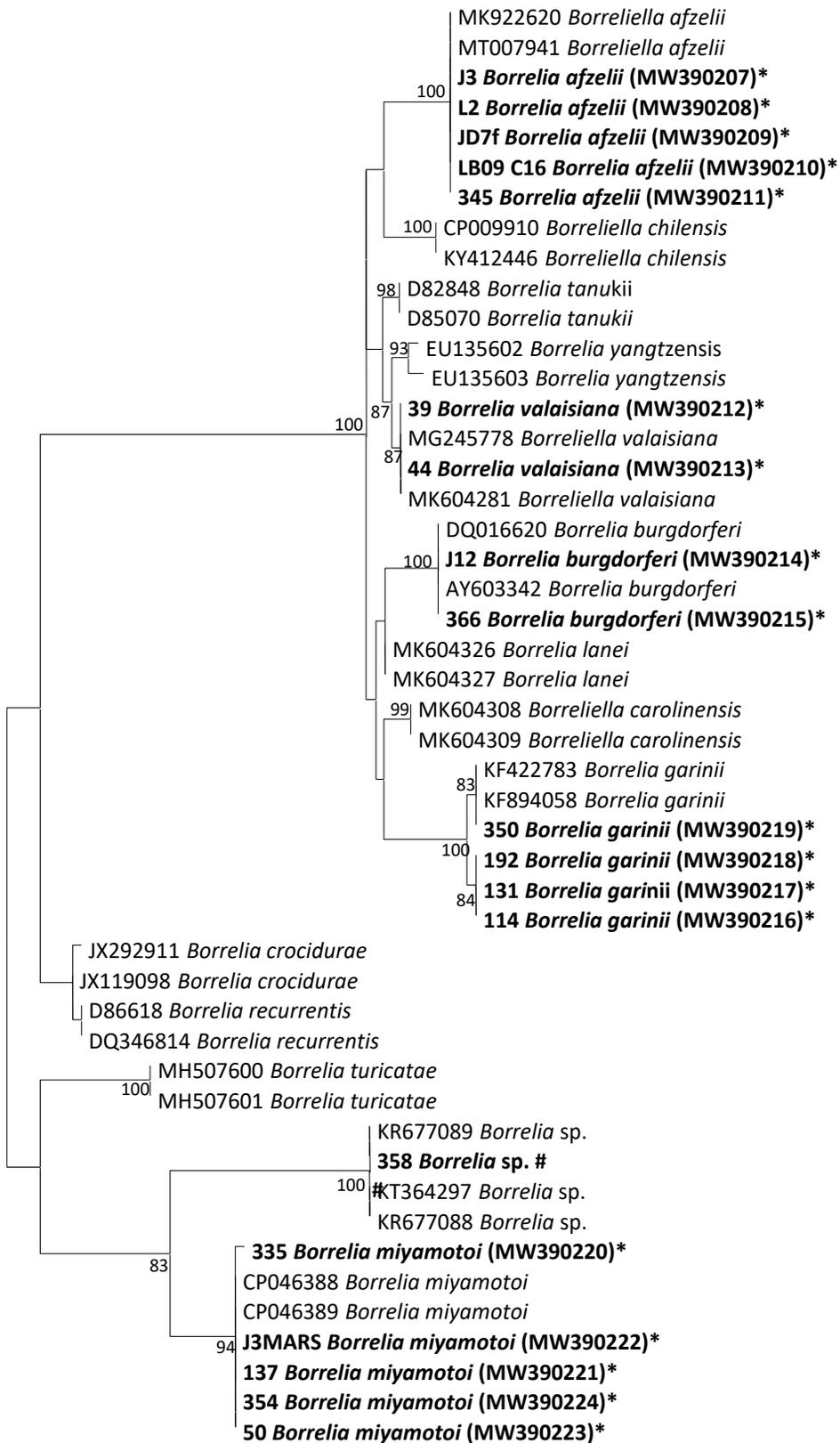
investigated in the Mercantour National Park, near Moulinet (43.9412878,7.4132216) in June 2020. For this eastern collect, altitudes ranged from 600m up to 1300m in height. The western Alpes-Maritimes were also investigated: the Loup Valley in the surroundings of Bar-Sur-Loup (43.7012408,6.988256). The locality of Courmes (43.742475,7.0087789) in March 2020 and the Caussols' plateau in the surroundings of Caussols Village (43.74242401123047,6.899637222290039) were also investigated. For this western part, altitudes ranged from 450m up to 1000m. Central Alpes-Maritimes were also surveyed in June 2020 in the Vésubie Valley, surrounding Levens-Village (43.8477377,7.2401105). In each location, ticks infected with human pathogens were collected. Numbers at the right of the pathogen's name indicate the number of infected ticks collected in this area. The letter P indicates when infected ticks originated from patients. The initials (I.r) stand for *I. ricinus* and (D.m) stand for *D. marginatus*.



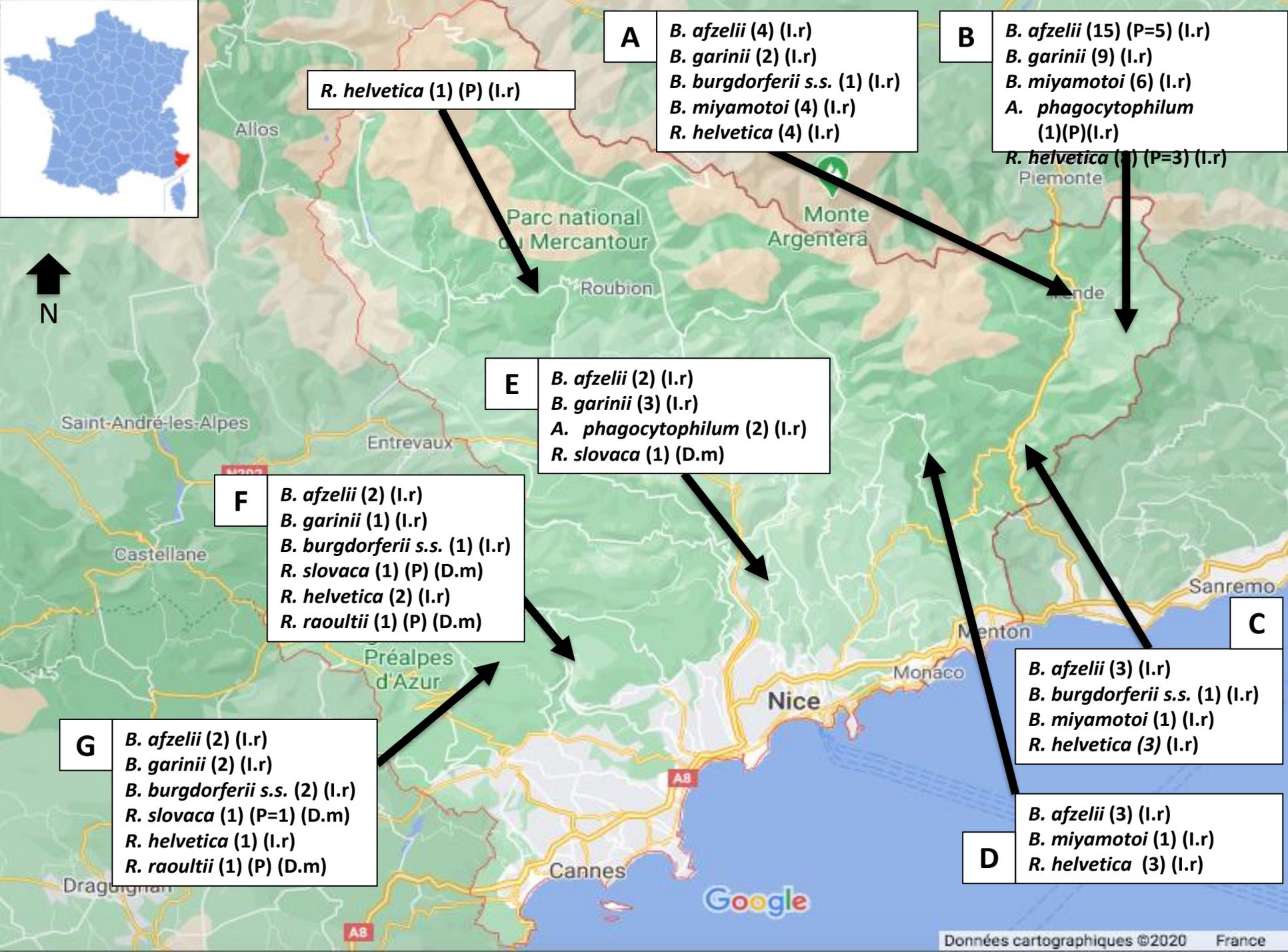
m/z



0.0050



0.050



R. helvetica (1) (P) (I.r)

A
B. afzelii (4) (I.r)
B. garinii (2) (I.r)
B. burgdorferii s.s. (1) (I.r)
B. miyamotoi (4) (I.r)
R. helvetica (4) (I.r)

B
B. afzelii (15) (P=5) (I.r)
B. garinii (9) (I.r)
B. miyamotoi (6) (I.r)
A. phagocytophilum (1)(P)(I.r)

R. helvetica (1) (P=3) (I.r)

E
B. afzelii (2) (I.r)
B. garinii (3) (I.r)
A. phagocytophilum (2) (I.r)
R. slovaca (1) (D.m)

F
B. afzelii (2) (I.r)
B. garinii (1) (I.r)
B. burgdorferii s.s. (1) (I.r)
R. slovaca (1) (P) (D.m)
R. helvetica (2) (I.r)
R. raoultii (1) (P) (D.m)

G
B. afzelii (2) (I.r)
B. garinii (2) (I.r)
B. burgdorferii s.s. (2) (I.r)
R. slovaca (1) (P=1) (D.m)
R. helvetica (1) (I.r)
R. raoultii (1) (P) (D.m)

C

B. afzelii (3) (I.r)
B. burgdorferii s.s. (1) (I.r)
B. miyamotoi (1) (I.r)
R. helvetica (3) (I.r)

D
B. afzelii (3) (I.r)
B. miyamotoi (1) (I.r)
R. helvetica (3) (I.r)