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Jean-Philippe Buffet

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## Doctorat ParisTech

# THÈSE

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le jeudi 6 décembre 2012

**Diversité des espèces de *Bartonella* circulant au sein d'une  
population de rongeurs sylvatiques dans une zone de forêt  
suburbaine, la forêt de Sénart**

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## Introduction générale

Durant ces vingt dernières années, un nombre croissant de bactéries appartenant au genre *Bartonella* a été identifié comme étant à l'origine d'une multitude de maladies humaines et animales, et plusieurs espèces de *Bartonella*, notamment *B. quintana*, sont considérées aujourd'hui comme des agents pathogènes émergents [1-2]. Ce constat a donc déclenché un intérêt considérable auprès des chercheurs à l'égard de ces bactéries gram-négatif et intracellulaires facultatives. L'intensification des études portant sur les espèces de *Bartonella* a permis de découvrir que ces microorganismes utilisent une stratégie unique pour coloniser leurs hôtes [2-4] et qu'elles sont capables d'infecter une vaste diversité d'espèces de mammifères, comprenant plusieurs ordres dont l'homme, des primates, des carnivores, des ongulés, des insectivores, des lagomorphes, des chiroptères et des rongeurs [5-6]. Il est maintenant admis que chaque espèce de *Bartonella* est adaptée à un ou quelques hôtes réservoirs [2, 5, 7] au sein desquels la bactérie établit une bactériémie intra-érythrocytaire pouvant persister plusieurs mois [3, 8]. La transmission de ces bactéries d'hôte à hôte est assurée par des vecteurs arthropodes hématophages compétents ou bien par contact direct avec un animal infecté [5].

Les espèces appartenant au genre *Bartonella* sont incluses dans le phylum des alpha-proteobacteria à proximité des bactéries du genre *Brucella* [9]. A ce jour, 33 espèces de *Bartonella* ont été recensées, et une vingtaine de ces espèces ont été isolées et/ou détectées à partir de rongeurs sauvages pour lesquels l'infection est asymptomatique [10-12]. Au moins 5 des espèces de *Bartonella* associées aux rongeurs (*B. grahamii*, *B. elizabethae*, *B. vinsonii* subsp. *arupensis*, *B. washoensis* et *B. tamiiae*) sont considérées comme des agents de zoonoses et l'infection chez l'homme peut conduire à des symptômes cliniques aigus espèces-dépendants, tels que des bactériémies, des fièvres, des myocardites et des endocardites [4].

D'après les données épidémiologiques apportées par plusieurs études du monde entier, les espèces de *Bartonella* adaptées aux rongeurs semblent être transmises d'hôte à hôte principalement par des puces, et, dans une moindre mesure, des tiques, des mites et des poux [13]. A l'heure actuelle, seule la compétence des puces *Ctenophthalmus nobilis nobilis* et *Xenopsylla ramesis* dans la

transmission d'espèces de *Bartonella* ont été clairement démontrées par des études expérimentales [14-15].

Les espèces de *Bartonella* infectant les rongeurs sont largement distribuées sur tous les continents (Amériques, Europe, Asie, Afrique, Moyen-Orient et Océanie, en excluant les pôles qui n'ont jamais été examinés), arborant des taux de prévalence et une diversité génétique élevés [16-21]. La présence d'une telle diversité au sein des communautés de rongeurs partout dans le monde est encore inexplicée. Néanmoins, de façon générale, toutes les espèces de *Bartonella* auraient évolué en s'adaptant à des facteurs écologiques liés à l'hôte, au vecteur arthropode et à l'environnement dans lequel l'hôte et le vecteur évoluent [5]. La spécificité d'hôte, le comportement de l'hôte, le statut immunitaire de l'hôte, la composition et la densité des hôtes, la compétence des vecteurs arthropodes vis-à-vis des espèces de *Bartonella*, la densité de vecteurs, ainsi que la présence d'autres microorganismes (bactéries, virus ou protozoaires) au sein des hôtes sont autant de facteurs qui semblent influencer la dynamique des espèces de *Bartonella* au sein des communautés de rongeurs sauvages et qui auraient, par le passé, façonné cette diversité.

L'objectif de cette thèse était d'élucider certains de ces aspects en étudiant la diversité des souches de *Bartonella* circulant au sein d'une population de rongeurs sylvatiques dans une zone de forêt suburbaine française, la forêt de Sénart (Ile-de-France).

En guise d'introduction générale de ce travail de thèse, nous avons donc entrepris d'écrire une revue bibliographique visant à retracer l'histoire naturelle des espèces de *Bartonella* inféodées aux rongeurs sauvages en établissant un état des lieux des données sur la diversité de ces espèces à travers le monde. Puis en récapitulant les connaissances actuelles concernant les interactions entre les espèces de *Bartonella* et leurs deux hôtes (les rongeurs et les vecteurs arthropodes), nous avons discuté les stratégies que les bartonellae ont élaborées pour s'adapter aux rongeurs.

Dans un second temps, nous avons mis en évidence l'association qui existe entre les souches de *Bartonella* et les espèces de rongeurs sylvatiques en Europe. En nous basant sur une phylogénie robuste, nous avons tout d'abord identifié les souches de *Bartonella* circulant au sein de la population de rongeurs composés de campagnols roussâtres (*Myodes glareolus*), de campagnols agrestes (*Microtus*

*agrestis*) et de mulots sylvestres (*Apodemus sylvaticus*). Pour construire cette phylogénie, nous avons amplifié et séquencé les fragments de 6 gènes ubiquistes (codant pour des protéines essentielles impliquées dans le métabolisme primaire de la bactérie) que nous avons ensuite concaténés, dans le but de discriminer distinctement les différentes souches de *Bartonella* isolées et détectées. Dans un premier temps, cette identification précise des souches de *Bartonella* nous a permis de déterminer leur niveau de spécificité vis-à-vis des espèces de rongeurs. Puis, dans un second temps, nous avons utilisés les séquences des 6 gènes de ménages pour détecter et quantifier les événements de recombinaison homologue entre les différentes souches de *Bartonella*. En utilisant les événements de recombinaison comme indicateur de co-infections, nous avons cherché à comprendre comment les différentes souches de *Bartonella* interagissent au sein des individus de la communauté de rongeurs.

Dans un troisième temps, nous avons contribué à la découverte des interactions qui peuvent se produire entre les espèces de *Bartonella* et d'autres microorganismes avec qui elles constituent une communauté écologique au sein des populations de rongeurs. Nous avons alors étudié l'impact que pourrait avoir la présence de *Borrelia afzelii*, une bactérie connue pour infecter fréquemment les rongeurs sylvatiques en Europe, sur la prévalence des espèces de *Bartonella*. Nous avons donc établi la prévalence et la diversité de *Borrelia afzelii* et des espèces de *Bartonella* au sein d'une population de campagnols roussâtres. Puis, en utilisant des tests statistiques, nous avons déterminé la fréquence des co-infections entre *Borrelia afzelii* et les souches de *Bartonella* au sein des individus afin d'observer d'éventuelles interactions entre les 2 genres bactériens.

Au cours de cette thèse, j'ai aussi participé à la détection et à l'identification d'autres espèces bactériennes vectorisées par des tiques et considérées comme des agents pathogènes émergents pour l'homme. J'ai participé à l'identification pour la première fois en France de *Candidatus Neoehrlichia mikurensis* au sein de campagnols roussâtres capturés dans la région des Ardennes en France. Ensuite, *Rickettsia monacensis*, *R. helvetica*, *Anaplasma phagocytophilum*, *Ehrlichia muris* et *Francisella tularensis* ont été détectées à partir de tiques collectées sur des chevreuils et des chèvres dans l'est de la Roumanie. Ces travaux ont fait l'objet de 2 publications scientifiques qui ont été ajoutées en annexe de cette thèse.

# **Chapitre I.**

## **Revue Bibliographique**

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Review to be submitted to Trends in Microbiology

**Natural history of *Bartonella* infecting rodents in the light of new  
knowledge on genomics, diversity and evolution**

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Key words : diversity, geographical structuration, arthropod vectors, adaptation

Running title : Bartonella in rodents

24 **Abstract**

25

26         The genus *Bartonella* comprises a unique group of facultative intracellular  
27 bacteria, highly adapted to a wide variety of mammals. Among the 33 validated  
28 *Bartonella* species to date, more than half are recognized to be hosted by rodent  
29 species, of which several have been involved in human illness. Data on *Bartonella*  
30 infection among rodent populations gathered from several countries and continents  
31 indicate a geographical structuration of *Bartonella* species in accordance with the  
32 localisation of their hosts. In all countries investigated, wild rodent species are  
33 infected by an extremely high diversity of *Bartonella* strains with high prevalence.  
34 *Bartonella* species have adapted to their rodent hosts in generating and maintaining  
35 this huge diversity, and arthropod vectors have a key role in the structuration of  
36 *Bartonella* species as well as the upholding of their diversity. Moreover, other  
37 microorganisms within rodents could interfere with the diversity of *Bartonella* species.  
38 In the present paper, we review the diversity of *Bartonella* species recovered from  
39 wild rodents worldwide and we discuss how they adapted to rodents.

40



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## 61 **I) Introduction**

62

63         Among wildlife, rodents are recognized as one of the major carriers of  
64 pathogens causing important diseases in livestock and human [1]. They are often  
65 implicated in the transmission of many bacteria to human causing zoonotic diseases,  
66 such as leptospirosis (*Leptospira* spp.), Lyme borreliosis (*Borrelia burgdorferi* s.l.) or  
67 plague (*Yersinia pestis*) [1-2]. Numerous gram-negative and facultative intracellular  
68 bacteria belonging to the genus *Bartonella* are known to infect mammals including  
69 rodents. Life cycle of *Bartonella* species has been elucidated from data obtained from  
70 both field studies [3] and laboratory infection models [4-5]. In the natural cycle of  
71 *Bartonella* species, arthropods play an essential role by serving as vectors and  
72 possibly as reservoirs [6]. It has long been reported that each *Bartonella* species are  
73 transmitted among their reservoir host by a specific arthropod species, such as  
74 *B. bacilliformis* vectored by the sand fly *Lutzomyia verrucarum*, *B. quintana* by the  
75 human louse *Pediculus humanus humanus*, or *B. henselae* by the cat flea  
76 *Ctenocephalides felis* [7-8]. However, experimental and epidemiological studies have  
77 demonstrated that some *Bartonella* species can be transmitted by several vector  
78 types [8]. For example, *B. henselae*, transmitted by the cat flea, has been detected in  
79 *Ixodes* ticks from several parts of the world [9], and the competence of *Ixodes ricinus*  
80 to transmit this *Bartonella* species has been proven by *in vivo* experiments [10].  
81 Overall, each *Bartonella* species are suspected to be transmitted by  
82 haematophagous arthropods to their mammalian host in which they infect a still  
83 unknown primary niche, probably formed by vascular endothelial cells [11]. After  
84 several days in the primary niche, they are seeded into the blood stream adhering to  
85 and invading erythrocytes. Once bartonellae colonize erythrocytes, they replicate in  
86 eight bacteria per cells and establish a long-lasting bacteremia until the natural death  
87 of erythrocytes [4]. In reservoir host, the infection is usually asymptomatic,  
88 *Bartonella* species being pathogenic when introduced into the wrong host (or into a  
89 immunosuppressed reservoir species).

90         Today, the genus *Bartonella* contains over 33 validated species, including at  
91 least 20 of them isolated and/or detected from wild rodents [12-13]. *Bartonella*  
92 rodent-associated species are distributed worldwide, exhibiting extremely high  
93 genetic diversity and prevalence that ranges from 6% in Greater Jakarta to 82% in

4

94 western Colorado (USA) [14-22]. Five of them have been recognized as human  
95 pathogens (*B. grahamii* [23], *B. elizabethae* [24-25], *B. vinsonii* subsp *arupensis* [26],  
96 *B. washoensis* [27] and *B. tamiiae* [28]), causing diverse and species-dependant  
97 symptoms, mostly bacteremia, myocarditis, endocarditis, lymphadenitis and hepatitis  
98 [29]. The importance of human disease caused by rodent-borne *Bartonella* species is  
99 still unknown. However, in the context where outdoor activities are growing, we can  
100 predict that the increasing possibilities of contact between humans (or their domestic  
101 animals) and wild rodents may lead to much more human diseases than those  
102 suspected.

103         In this review, we propose to provide an overview of what is known about the  
104 natural history of rodent adapted *Bartonella* species under progress made on the  
105 genomic, diversity and evolution aspects of these bacteria. Firstly, we trace the  
106 history of the discovery of rodent associated *Bartonella* from the year of the discovery  
107 of the first 3 species (1994) until today. In a second part, we explore their diversity  
108 according to their host and their geographical localisation to identify how they have  
109 evolved; thirdly, we investigate how they interact with their hosts (mammalian one  
110 and vectors one) and the strategies they elaborated for adaptation to their rodent  
111 hosts. In a fourth part, we investigate how they interact with other microorganisms  
112 they can encounter within the mammalian host and how their diversity could interfere  
113 with those interactions. This overview of the literature will bring new insights on the  
114 evolution of rodent associated *Bartonella* species.

115

116 **II) History of the discovery of rodent associated *Bartonella***  
117 **species**

118

119 The first *Bartonella* species to be identified was the human deadly pathogen  
120 *Bartonella bacilliformis* in south America in 1928. At the same period in Europe, a  
121 bacteria responsible of quintane fever was characterized in soldiers from the world  
122 war I. This bacteria was named *Rochalimae quintana* in the 60's. The medical  
123 interest for *B. quintana* became scarce until it has re-emerge in Europe in the 90's in  
124 homeless [30]. In the 90's, the agent of cat scratch disease was isolated for the first  
125 time from a human case and was named *Rochalimae henselae* [30] while Birtles et  
126 al. isolated at the same time three bacteria species affiliated to the *Grahamella* genus  
127 from the blood of small woodland rodents (voles and mice) in UK [31]. Since, with the  
128 advent of molecular techniques, the *Grahamella*, *Bartonella* and *Rochalimae* genera  
129 were unified in one genus, the so-called *Bartonella* genus.

130

131 **1- Rodent associated *Bartonella* species discovery**

132

133 How to identify *Bartonella* species?

134 For many years in bacterial systematic, the accepted species definition was  
135 that strains, with a homology greater than 70% when performed DNA-DNA  
136 hybridization, and a  $\Delta Tm$  of 5°C or less, belong to the same species. Below the value  
137 of 70% homology, strains were considered as belonging to different species [32].  
138 However DNA-DNA hybridization requires special equipment restricted to few  
139 laboratories worldwide, and this technique remains very difficult to perform specially  
140 in the case of *Bartonella* for which the number of species belonging to a same  
141 genera is consistently growing. An alternative method is the sequencing of the  
142 16SrRNA locus, used in the 70s to delineate the three domains of life [33]. In 1996,  
143 phylogenetic analysis based on 16SrRNA sequences discriminated successfully  
144 *B. henselae*, *B. quintana* and *B. bacilliformis* [34], but was not sufficient to distinguish  
145 more closely related *Bartonella* species [35]. Then, in 1995, Norman and colleagues  
146 proposed to use a fragment of the housekeeping gene *gltA* (encoding for the citrate  
147 synthase) to identify related *Bartonella* species [36], of which the phylogeny was

148 demonstrated to be more appropriate for investigating genetic relationships between  
149 *Bartonella* species than that based on 16SrRNA gene [37].

150 It is now admitted that the 16SrDNA sequence is sufficient to identify  
151 *Bartonella* at the genera level. Other protein-encoding genes than *gltA*, such as *rpoB*  
152 [38], *groEL* [39], *ftsZ* [40] and *ribC* [41], and *ialB* [42] were tested to identify  
153 *Bartonella* at the species level. La Scola et al. undertook to compare seven loci  
154 (16SrRNA, *gltA*, *rpoB*, *groEL*, *ftsZ*, *ribC* and the internal transcribed spacer (ITS)) for  
155 their ability to delineate 17 *Bartonella* species and subspecies, and they found that  
156 *rpoB* and *gltA* were the most potent for distinguishing clearly each species and  
157 subspecies [35]. Since then, the sequencing of *gltA* gene became a widely used tool,  
158 and a plethora of studies focused on it to describe *Bartonella* diversity in wildlife.  
159 However homologous recombination events can occur within the *gltA* gene even  
160 leading to some species misidentification [19]. Today, for description of new  
161 *Bartonella* species, MLSA analysis enabled to replace efficiently the DNA-DNA  
162 hybridization method [43-44]. Indeed, the criterion of 70% DNA-DNA reassociation  
163 for bacterial species demarcation is in accordance with an average nucleotidic  
164 divergence of 5% within housekeeping genes [45].

165 For intra-species investigation of bartonellae, a recent study revealed that the  
166 use of *gltA* is limited to resolve phylogenetic relationships between closely related  
167 *Bartonella* genotypes, because homologous recombination events can occur, biasing  
168 a precise identification of certain genotypes [19]. One way to overcome the issue of  
169 recombination is to use multiple genes scattered throughout the genome to construct  
170 a robust phylogeny. The multi-locus sequence analysis (MLSA) allows to build robust  
171 and informative phylogenies concatenating the sequences of multiple genes  
172 sequenced from strains of closely related species. The MLSA approach has  
173 improved the discrimination of closely related *Bartonella* genotypes, especially in  
174 rodent populations [46-48] in which lateral gene transfers has been found to be  
175 relatively frequent [19, 49-50]. Unfortunately, MLSA analysis is not sufficiently spread  
176 in the *Bartonella* community, and many studies keep identifying *Bartonella* species  
177 using only the *gltA* gene, leading to endless lists of DNA sequences ambiguously  
178 assigned to uncertain *Bartonella* species or to hypothetical new species.

179

180

181

182 What are the validated rodent-associated *Bartonella* species in 2012?

183 After the isolation in 1994 of three bacteria species affiliated to the *Grahamella*  
184 genus from the blood of small woodland rodents (voles and mice) in UK [31], Birtles  
185 et al. unified the *Grahamella* and *Bartonella* genera using 16SrRNA based phylogeny  
186 and DNA-DNA hybridization and described 5 rodent associated Bartonella species:  
187 *B. taylorii*, *B. doshiae* and *B. grahamii*, *B. talpae*, *B. peromysci* [51]. In the same time,  
188 a strain isolated from a vole trapped in Canada in 1946 and identified as *Rickettsia*  
189 *prohwzeki* was renamed *B. vinsonii* on the basis of 16SrRNA analysis [52]. Because of  
190 its close relatedness to a strain isolated soon after from the blood of a dog, this  
191 species was subdivided into 2 subspecies, and the vole strain was then called  
192 *B. vinsonii* subsp. *vinsonii*, while the dog strain was named *B. vinsonii* subsp.  
193 *berkhofii* [53]. In 1999, a novel subspecies of *B. vinsonii* was isolated from the blood  
194 of a cattle rancher in Wyoming with bacteraemia and fever, and Welch et al.  
195 proposed the name *B. vinsonii* subsp. *arupensis* for this strain [26]. A later study  
196 revealed that *B. vinsonii* subsp. *arupensis* infects deer mice with high prevalence  
197 across North America [14]. In 1999, *B. tribocorum*, was isolated from wild rats (*Rattus*  
198 *norvegicus*) inhabiting a rural environment in France [54]. In the 2000's, several other  
199 rodent-adapted species has been isolated worldwide, in a plethora of rodent species:  
200 *B. birtlesii* in wood mouse (*Apodemus* spp.) from France [55]; *B. rattimassiliensis* and  
201 *B. phoceensis* in *Rattus norvegicus* from France [56], *B. queenslandensis*,  
202 *B. rattaaustraliani* and *B. coopersplainsensis* in wild rats from Australia [57],  
203 *B. japonica* and *B. sylvatica* from field mouse species in Japan [48], and, in 2012, *B.*  
204 *jaculi*, *B. callosciuri*, *B. pachyuromydis* and *B. acomydis* from wild-captive rodents  
205 originally imported in Japan as pets from Egypt, Thailand and the Netherlands [58].

206 Some of the rodent adapted *Bartonella* species have been firstly isolated from  
207 human cases, their rodent origins being determined subsequently. This is the case  
208 for *B. elizabethae*, an agent isolated in a human case of endocarditis in North  
209 America [24] and then, identified in wild rodents in Peru and North America [37, 59];  
210 *B. washoensis* discovered in a human case presenting fever and myocarditis in USA,  
211 and, then found to infect ground squirrels [27]; *B. rochalimae*, involved in human  
212 infection in US [60] and then isolated from wild rats (*Rattus norvegicus*) in Taiwan  
213 [61]. *B. tamiiae*, a newly human identified pathogen, isolated from blood samples  
214 collected on patients in northeast Thailand [28] was detected in ectoparasites  
215 isolated from different rodent species suggesting the role of rodents in its life cycle



216 [12]. All the different rodent species identified so far, their identified reservoir, vector  
217 and their potential as human pathogens are listed in Table 1.

218

219 **2- Actual taxonomy of *Bartonella* and phylogenetic position of the rodent**  
220 **associated species**

221

222 The *Bartonella* genus belongs to the alpha proteobacteria phylum in proximity  
223 to the *Brucella* genus. Both genera are classified in the family Rhizobiales which also  
224 comprises numerous taxa of plant-associated and environmental bacteria, such as  
225 Rhizobiaceae (e.g. *Agrobacterium tumefaciens*) and Methylobacteriaceae (e.g.  
226 *Methylobacterium* spp.). Using the approaches cited above, 33 *Bartonella* species  
227 have been validly characterized to date, *Bartonella vinsonii*, subdivided into three  
228 subspecies.

229 Inference of the phylogenetic relationships within the genus *Bartonella*  
230 revealed five major clades or lineages [62-63] (Figure 1). The lineage 1 is only  
231 represented by the newly discovered *B. tamiae*, which discloses a profound  
232 divergence with other *Bartonella* species. *B. bacilliformis* is the sole representative of  
233 lineage 2 and was considered as the ancestor of the remaining lineages. Lineage 3 is  
234 composed of the four ruminant-specific *Bartonella* species (*B. bovis*,  
235 *B. schoenbuchensis*, *B. capreoli* and *B. chomelii*), and lineage 4 is represented by  
236 *B. clarridgeiae*, isolated from cats, and *B. rochalimae*, isolated from dog, foxes and  
237 rodents. Lineage 5 is the most species-rich clades comprising the remaining 23  
238 *Bartonella* species (Figure1). Lineages 4 and 5 are ramifying to different degrees and  
239 include species recovered from various mammal hosts. It has been proposed that the  
240 diversification of species within these two lineages is due to a specific adaptation to  
241 distinct mammalian host, the so-called adaptive radiation [63]. The *Bartonella* rodent-  
242 associated species are almost all included in the lineage 5, except *B. rochalimae*  
243 (lineage 3). Species included in lineage 5 represent the most frequently found  
244 *Bartonella* species in natural host populations, which is not surprising given that  
245 Rodentia is the most abundant and diversified order of living mammals, representing  
246 about 43% of the total number of mammalian species [64].

247

248 **III) Structuration of *Bartonella* according to the geographical**  
249 **localisation of their hosts**

250

251 During the last decade, data on *Bartonella* infection among rodents  
252 continuously increased. The geographical information gathered in different countries  
253 and continents strongly indicated that *Bartonella* species are structured according to  
254 the localisation of their rodent host. Consequently, according to the distribution of  
255 rodent hosts, some rodent adapted *Bartonella* species are globally distributed, while  
256 others are highly adapted to rodents inhabiting specific biotopes.

257

258 **1- *Bartonella* species recovered worldwide**

259 The most investigated rodent host for *Bartonella* species is *Rattus* rats (*Rattus*  
260 *rattus* and *Rattus norvegicus*) which have a worldwide distribution due to increased  
261 trade and favourable living conditions created by human presence  
262 (www.iucnredlist.org). Several *Bartonella* species have been found to infect  
263 frequently *Rattus* rats: *B. elizabethae*, *B. tribocorum*, *B. phoceensis*,  
264 *B. rattimassiliensis*, *B. queenslandensis*, *B. coopersplainsensis*, and *B. rochalimae*.  
265 *B. elizabethae*, *B. tribocorum*, *B. rattimassiliensis* and *B. queenslandensis* are  
266 clustered in a well-demarcated branch in proximity to *B. grahamii* (Figure 1), which  
267 represent the so-called *B. elizabethae*-like species. All studies conducted in North  
268 and South America, Europe, Asia, Middle East, and Africa reported infection of  
269 *Rattus* rats by these *B. elizabethae*-like species, *B. phoceensis* and  
270 *B. coopersplainsensis* [15, 20, 50, 54, 56, 59, 65-75].

271 Other genera of rats, mice, shrews and gerbils were found to be infected by  
272 *B. elizabethae*-like species, such as *Bandicota* rats and *Suncus murinus* in Asia, or  
273 Namaqua Rock rats (*Aethomys namaquensis*) and Bushveld gerbil (*Tatera*  
274 *leucogaster*) in South Africa [71, 76]. This broad host range of *B. elizabethae*-like  
275 species can be explained by a common vector community which infests these rodent  
276 species. Indeed, some epidemiological studies performed in North Africa, Middle  
277 East, China and North America detected *B. elizabethae*, *B. tribocorum* and  
278 *B. queenslandensis* DNA in *Xenopsylla* fleas infesting *Rattus* and *Bandicota* rats,  
279 gerbils, *Mus* mice and shrews [70, 77-79].

280



281 Comparing the carriage of *Bartonella* by rat species reported by all the studies  
282 cited above, the diversity of *Bartonella* species seems to be higher in southern Asia  
283 than anywhere else. For example, in Thailand, the majority of *Bartonella* strains  
284 infecting *Rattus* rats belongs to 3 *B. elizabethae*-like species, *B. coopersplainensis*  
285 and *B. phoceensis* [67], while *Rattus* rats in Los Angeles host *Bartonella* strains  
286 mainly affiliated to 3 species (*B. tribocorum*, *B. rochalimae* and *B. queenslandensis*)  
287 [65]. Furthermore, the number of host species carrying *B. elizabethae*-like species is  
288 also higher in southern Asia, such as *Bandicota indica*, *Mus cervicolor*, 5 different  
289 *Rattus* rat species, ... [67, 71] than the remaining continents. A possible explanation  
290 to the higher diversity of *B. elizabethae*-like species observed in southern Asia as  
291 compared with those in other continents is that *B. elizabethae*-like species appeared  
292 first in Asia and then spread in other countries by the introduction of *Rattus* rats [80].

293 The description of the transmission routes of *Bartonella* species, i.e. the age of  
294 *Bartonella* infection in rodents, where the ancestor of a *Bartonella* species are  
295 originated and how it has migrated since then, is of particular interest for  
296 understanding the high diversity and prevalence of infection among rodents. Until  
297 now, only two studies on *Bartonella* infection in rat species investigated the origin of  
298 *B. elizabethae*-like. Based on *Bartonella gltA* sequences obtained from *Rattus* rats  
299 collected in Europe and America, Ellis et al. [59] hypothesized that *B. elizabethae*  
300 strains are originated from the Old World (Europe), and have been introduced in the  
301 New World (America) by the importation of rats. Investigating *Bartonella* infection of  
302 rodents in China, Ying et al. demonstrated that *Bartonella* strains previously  
303 described in rats from America and Europe are representatives of the diversity of a  
304 very large group of *Bartonella* species appearing to be strongly associated with  
305 *Rattus* rats [16].

306 A recent detailed study on the historical transmission routes of the agent of  
307 plague, *Yersinia pestis*, hosted by *Rattus* rats as reservoir, showed that *Y. pestis*  
308 strains evolved in or near China, and then spread through multiple radiations to  
309 Europe, South America, North America, Africa and Southeast Asia. As for *Y. pestis*, it  
310 would be interesting to establish the origin of *B. elizabethae*-like species using robust  
311 typing methods and to know whether the diversification of *B. elizabethae*-like species  
312 followed similar trajectories as *Yersinia pestis* [80].

313  
314

315 **2- *Bartonella* species adapted to sylvatic rodents in boreal forests of the**  
316 **northern Hemisphere**

317 **\**B. grahamii*, *B. taylorii*, *B. doshiae* and *B. birtlesii***

318 With the exception of one strain isolated from the blood of a bushveld gerbil  
319 (*Tatera leucogaster*) in South Africa and one other strain isolated from a *Rattus* rat in  
320 Peru, *B. grahamii* strains have been found to be widespread among *Myodes* and  
321 *Microtus* voles in the northern Hemisphere (North America, Europe and Asia),  
322 *Apodemus* mice in Eurasia and grasshopper mice (*Onychomys leucogaster*) in North  
323 America [46, 50, 76, 81-84]. MLSA analysis of *B. grahamii* strains sampled worldwide  
324 revealed a strong geographic pattern and high level of gene content and sequence  
325 divergence compared to *Bartonella* species inferred to cat (*B. henselae*) and human  
326 (*B. quintana*) [46, 49]. Berglund et al. explained these differences by different  
327 migration pattern of the hosts [49]. Indeed, *B. grahamii* strains are mainly recovered  
328 from mice and voles which migrate slowly and typically have a home area of less  
329 than 1 ha, while domestic cats and humans travel much longer distances. Based on  
330 *gltA* phylogeny, Inoue et al. suggested that *B. grahamii* existed in North America  
331 before Europe, and crossed the Bering's strait during a glacial epoch to the Eurasian  
332 continent [46]. However, this analysis was performed on the basis of *gltA* gene only  
333 which is now known to be an unreliable tool to infer precise phylogenies. The  
334 representation of the diversity of *B. grahamii* strains used in these studies was  
335 probably underestimated and should be re-evaluated using MLSA analysis [49].

336 Such as *B. grahamii*, *B. taylorii* also exhibits high diversity and is frequently  
337 found in *Apodemus* mice and *Myodes* and *Microtus* voles inhabiting boreal forests of  
338 the Eurasian continent only [31, 50, 82, 85]. However, the transmission routes of  
339 *B. taylorii* strains circulating in Eurasian mice and voles remain to elucidate.

340 *B. doshiae* strains are commonly recovered from mice and voles in Europe  
341 [19, 31, 83, 86-87] and in cotton rats (*Sigmodon hispidus*) in USA [17, 88], while that  
342 of *B. birtlesii* have been found to be distributed among mice and voles only in Europe  
343 [19, 55, 83, 87]. In contrast to *B. grahamii* and *B. taylorii* and according to all studies  
344 cited above, *B. doshiae* and *B. birtlesii* exhibited very low prevalence and genetic  
345 diversity, indicating that these species are probably enduring a bottleneck (i.e.,  
346 reduction of population size). This bottleneck phenomenon can be attributed to either  
347 a relatively recent host shift or a restriction of these species to few hosts or vectors.

348

349           **\**B. vinsonii* subsp. *arupensis*, *B. vinsonii* subsp. *vinsonii* and *B. vinsonii***  
350 **subsp. *berkhoffii***

351           These three *Bartonella* subspecies have been found to be widespread among  
352 sylvatic rodents in North America.

353           Genotypes related to *B. vinsonii* subsp. *arupensis* were recovered from the  
354 blood of rodent species belonging to *Peromyscus* and *Onychomys* genera with very  
355 high prevalence (up to 82% in deer mice) in nearly all the states of USA and in  
356 Canada [14, 17, 27, 81, 84, 89].

357           *B. vinsonii* subsp. *vinsonii* strains were also isolated with high prevalence  
358 (68%) in woodrats (*Neotoma* sp.) in New Mexico (USA) and to a lesser extent in red-  
359 backed voles (*Myodes gapperi*) and meadow voles (*Microtus pennsylvanicus*) in  
360 Canada [52, 81, 90].

361           *B. vinsonii* subsp. *berkhoffii*, a species frequently found in the blood of dogs  
362 with endocarditis in US [91], have been recovered in eastern states from cotton rats  
363 (*Sigmodon hispidus*) [17, 88]. In addition, these cotton rats hosted also several  
364 strains related to the *B. vinsonii* subspecies complex, representing a divergent  
365 lineage from the three known *B. vinsonii* subspecies [88].

366           Besides North America, there are some evidences of the presence of the 3  
367 species in other continents. In Thailand, strains closely related to *B. vinsonii* subsp.  
368 *arupensis* have been detected in human cases of illness and represented the most  
369 frequently found *Bartonella* species in the blood of stray dogs [92-93]; and strains,  
370 exhibiting 100% 16SrRNA gene sequence similarity to *B. vinsonii* subsp. *vinsonii*  
371 type strain, have been recovered from mice and voles captured in Denmark [94].

372

373           **\**B. washoensis***

374           Almost all *Bartonella* isolates inferred to ground sciurid species in USA and  
375 China, including ground squirrels of the genus *Spermophilus*, prairie dogs of the  
376 genus *Cynomys*, and chipmunks of the genus *Tamias* were shown to be related to  
377 the species *B. washoensis*. Inoue et al. demonstrated, using a MLSA analysis, that  
378 *B. washoensis* strains have co-speciated with their squirrel hosts. Interestingly, within  
379 sciurid genera, genotypes found in China are divergent from American ones [47].  
380 Currently, there is no data about the carriage of *Bartonella* species in Sciuridae from  
381 Europe or other part of the world.

382

383 **3- *Bartonella* rodent-associated species of the southern Hemisphere**

384 Even few reports described rodent-adapted *Bartonella* species in the southern  
385 Hemisphere, it appears that many new *Bartonella* species yet to be discovered  
386 circulate in this part of the world.

387 The most detailed study was done in South Africa and revealed several strains  
388 probably belonging to a novel *Bartonella* species and isolated from the blood of  
389 striped mice (*Rhabdomys pumilio*), Namaqua rock rats (*Aethomys namaquensis*) and  
390 Natal multimammate mice (*Mastomys natalensis*) [76]. The authors hypothesized  
391 that these strains were probably highly adapted to South African rodents [76].  
392 Similarly, strains representing potentially new *Bartonella* species have been found in  
393 *Mus* mice and in *Lophuromys* rats in sub-Saharan Africa [95].

394 In South America, despite the history of one of the most deadly pathogen  
395 *B. bacilliformis*, data about *Bartonella* infection in rodents are very restricted. The  
396 only study analyzing *Bartonella* in rodents dates from 1996 and revealed, four  
397 isolates of an unidentified *Bartonella* species obtained from leaf-eared mice (*Phyllotis*  
398 spp.) trapped in Peru [37].

399

400

401

#### 402 **IV) Strategies of adaptation of *Bartonella* species to rodents**

403

404 All studies on *Bartonella* species within rodent communities in different parts of  
405 the world indicate high rates of prevalence and genetic diversity. In order to maintain  
406 such high prevalence and diversity in natural rodent populations, *Bartonella* species  
407 have evolved elaborating several strategies to generate diversity, and the other hosts  
408 of *Bartonella* species, the arthropod vectors, appear to have a major role in the  
409 diversification of *Bartonella* strains by promoting recombination.

410

##### 411 **1- Association between *Bartonella* and its rodent host**

412 According to field studies, the specificity of association between *Bartonella*  
413 and its rodent hosts might depend on *Bartonella* species and their geographical  
414 origin. *B. elizabethae*, *B. tribocorum*, *B. rattimassiliensis*, *B. phoceensis*,  
415 *B. queenslandensis* and *B. coopersplainsis* are highly specific to *Rattus* rats and  
416 *Bandicota* rats, shrews and gerbils [16, 65, 69, 71], while *B. vinsonii* subsp. *arupensis*  
417 is hosted as a reservoir by *Peromyscus* and *Onychomys* mice in USA [14, 17, 90].  
418 Using a MLSA approach, it has been demonstrated that strains related to  
419 *B. washoensis* are specifically adapted to their squirrel hosts (*Spermophilus* and  
420 *Tamias* species) in North America and China [47].

421 The association between the other *Bartonella* species and their rodent hosts is  
422 still conflicting. In Europe, *B. taylorii*, *B. grahamii* and *B. doshiae* could infect several  
423 sympatric woodland rodents at a given site [21, 83, 96]. The MLSA analysis of  
424 several *B. grahamii* strains sampled worldwide showed a pattern of strict host  
425 specificity in Asia and North America, compared to lowest host specificity in Europe  
426 [46]. However, a longitudinal investigation of the infection by *B. grahamii*, *B. taylorii*,  
427 *B. birtlesii* and *B. doshiae* within a population composed of wood mice (*Apodemus*  
428 *sylvaticus*) and bank voles (*Myodes glareolus*) in UK highlighted that *B. doshiae* and  
429 *B. taylorii*, were more prevalent in wood mice, while *B. birtlesii* was mostly present in  
430 bank voles suggesting a certain host specificity [97]. These observations are  
431 supported by a more recent long-term study realized in Poland reporting a strong  
432 host-association between the majority of *B. taylorii* and *B. grahamii* genotypes and  
433 yellow-necked mice (*Apodemus flavicollis*) and bank voles (*Myodes glareolus*) [98].

15



434 Recently, using several housekeeping genes, we realized a robust phylogeny to  
435 discriminate accurately strains belonging to *B. taylorii*, *B. grahamii* and *B. doshiae*  
436 isolated from a rodent community composed of bank voles, field voles and wood  
437 mice in France, and we demonstrated that almost all strains, at the level of genotype,  
438 are highly specific to either bank voles or wood mice [Buffet et al., manuscript  
439 submitted].

440 Considering molecular mechanisms, by *in vivo* and *in vitro* experiments with  
441 phylogenetically distant bartonella species (*B. birtlesii*, *B. henselae*, *B. quintana* and  
442 *B. tribocorum*) infecting phylogenetically distant mammalian host (mouse, rat, cat and  
443 human) we have shown that the host specificity of bartonellae arises from their ability  
444 to adhere to erythrocytes of their natural host(s) [99]. At the molecular level,  
445 *Bartonella* host-specific adhesion is mediated by the Trw type 4 secretion system  
446 (Trw T4SS) [99]. So it would be interesting to test, using the same *in vivo* and *in vitro*  
447 methods, the host specificity of *Bartonella* strains isolated from wild rodent  
448 populations.

449

## 450 **2- Transmission of *Bartonella* species among rodent populations**

451 Among rodent populations, the transmission of *Bartonella* species appears to  
452 be mainly ensured by fleas. The rodent flea *Ctenophthalmus nobiles* is a competent  
453 vector for *B. grahamii* and *B. taylorii* among bank voles (*Myodes glareolus*) in UK  
454 [100]. Similarly, *Bartonella* genotypes circulating within gerbilline rodents are  
455 transmitted by *Xenopsylla ramesis* in the deserts of Middle East [101]. Otherwise,  
456 indirect evidences of transmission of *Bartonella* species among rodents by fleas, lice,  
457 mites and ticks have been provided by DNA footprint of *Bartonella* in natural  
458 populations (see the review of Tsai et al. [9]).

459 Using *in vivo* experiments, the *Ixodes ricinus* tick have been also shown to be  
460 competent for the transmission of *B. birtlesii* [102]. However, in UK, the abundance of  
461 *Ixodes ricinus* ticks on small mammals did not affect the probability of wood mice  
462 (*Apodemus sylvaticus*) being infected by *Bartonella* species suggesting that *Ixodes*  
463 *ricinus* is not an epidemiologically relevant vector of *Bartonella* species among  
464 woodland rodents [103].

465

466

467

468 **3- Adaptation of *Bartonella* species to rodents**

469 The high diversity of *Bartonella* species in rodent populations apparently  
470 contributes to high prevalence of infection in animals since it allows these bacteria to  
471 avoid the impact of host immune response to a particular strain. This idea is  
472 supported by long-term studies realized in Europe and North America, with a mark-  
473 recapture strategy, showing that rodent species remain infected by these  
474 microorganisms over a long period of their life, with different *Bartonella* variants,  
475 replacing each other [98, 104-105]. So generating diversity appears to be a common  
476 strategy for *Bartonella* species adapted to rodents allowing to escape the host  
477 immune system.

478

479 How do *Bartonella* strains generate diversity?

480 It is now admitted that the diversification of bacterial species occurs mainly by  
481 homologous recombination than *de novo* mutation, however this phenomenon  
482 remains scarce when the genetic distance increased between species [106]. In the  
483 particular case of *Bartonella* species, recombination events at genes coding for host  
484 adaptability factors (i.e. type IV secretion systems) have been demonstrated to be  
485 driven by selection [63, 107-108].

486 The comparison of the complete genome sequences between two *Bartonella*  
487 species hosted by rodents (i.e. *B. tribocorum* and *B. grahamii* strains) and *Bartonella*  
488 species inferred to human and cat revealed that the rodent-associated species own a  
489 higher amount of genes coding for host-adaptability factors than the other species  
490 [107]. Interestingly, these host-adaptability coding genes are packaged into  
491 bacteriophage particles, generating an original fashion of genes exchange between  
492 *B. grahamii* strains, and thus promoting rapid diversification. This system could  
493 therefore facilitate host shift despite the host specificity [107]. Furthermore, Berglund  
494 et al. showed that the contribution of homologous recombination in generating  
495 diversification among *B. grahamii* strains sampled worldwide was significantly higher  
496 than *B. henselae* [49]. Recently, we confirmed the high contribution of lateral gene  
497 transfers in generating diversity among *B. grahamii* strains, and we demonstrated  
498 that *B. taylorii* undergoes even more frequent homologous recombination events,  
499 suggesting that *B. taylorii* owns a similar system to that of *B. grahamii* in its genome  
500 [Buffet et al., manuscript submitted]. As demonstrated previously in Poland [19], we  
501 also noted that *B. grahamii* and *B. taylorii* strains can exchange genetic materials

502 suggesting that the combination of these two species in sylvatic rodent communities  
503 enhances their diversification.

504

505 Where can *Bartonella* strains exchange genetic material?

506 Firstly, mixed infections of multiple *Bartonella* strains have already been  
507 observed within rodents [109]. Moreover, the longitudinal study of Bai and colleagues  
508 underlay spillover for *Bartonella* species [84]. Spillover is a 'jump' phenomenon of a  
509 parasite from its reservoir host to a non-specific one, which occurs only when the  
510 prevalence in the donor host reaches a threshold [12]. In the western states of US,  
511 northern grasshopper mice (*Onychomys leucogaster*) are frequently infected by  
512 specific *B. vinsonii* subsp. *arupensis* strains. But Bai et al. [84] demonstrated that  
513 some *B. vinsonii* subsp. *arupensis* strains, adapted to deer mice (*Peromyscus*  
514 *maniculatus*), can be acquired by northern grasshopper mice. *Bartonella* jumps  
515 between several rodent host species have been also observed in Thailand,  
516 identifying a strain in a *Rattus rattus* nearly identical to strains hosted by *Bandicota*  
517 *indica* [66]. This 'jump' phenomenon has been demonstrated by experimental  
518 infections of Swiss Webster mice by a *B. elizabethae* strain originally isolated from an  
519 Asian house shrew in Vietnam. Laboratory mice acquired the *B. elizabethae* strain  
520 developing a bacteraemia, only after inoculation of high doses of bacteria [110]. So  
521 spillover would allow the co-presence of closely related but divergent *Bartonella*  
522 strains, and thus promotes recombination.

523 Secondly, through their competence towards the transmission of *Bartonella*  
524 species, arthropod vectors have a role in the selection of *Bartonella* genotypes [6].  
525 Moreover, the arthropod vector seems to have a key role in the upholding of the  
526 diversity of *Bartonella* species in rodents by promoting lateral gene transfers among  
527 strains. Investigating *Polygenis gwyni* fleas infesting cotton rats (*Sigmodon hispidus*)  
528 in Georgia (USA), Abbot et al. [88] showed mixed infections of these fleas by  
529 different *Bartonella* genotypes with an extremely high rate (90%). They detected  
530 mainly *Bartonella* genotypes known to be hosted by cotton rats in Southern states of  
531 US (e.g. *B. vinsonii* subsp. *arupensis*), and atypical *Bartonella* genotypes related to  
532 *B. clarridgeiae* and *B. rochalimae* [88]. Hence, the co-presence of different *Bartonella*  
533 strains within vectors present opportunities for lateral gene transfers. These  
534 mechanisms may have a key role in the diversification as well as the selection of  
535 *Bartonella* strains [12, 88].



536 Similarly, in Colorado, some fleas (*Oropsylla hirsuta*, *Aetheca wagneri* and  
537 *Orchopeas leucopus*) collected on prairie dogs (*Cynomys ludovicianus*) and deer  
538 mice (*Peromyscus maniculatus*) carried atypical *Bartonella* species [111].  
539 *B. washoensis* hosted by prairie dogs [112] was found in deer mice fleas, and,  
540 inversely, *B. vinsonii* subsp. *arupensis* DNA was found in fleas infesting prairie dogs  
541 [111]. Afterwards, the majority of *Bartonella* species identified in rats (*Rattus*  
542 *norvegicus*) and their fleas (*Xenopsylla cheopis*) from downtown Los Angeles were  
543 identical and belonged to *B. tribocorum*, except some fleas carrying *B. rochalimae*  
544 [78]. Thus, coinfection by different *Bartonella* strains and presence of atypical strains  
545 within the vector may explain the 'jump' phenomenon of non-specific strains between  
546 hosts observed in Colorado and Asia [66, 84]. Moreover, some flea species can be  
547 shared between several rodent species. For example, grasshopper mice and deer  
548 mice can be both infested by *Aetheca wagneri*, *Orchopeas leucopus* and *Pleochaetis*  
549 *exilis*. Grasshopper mice are known to usurp the burrow of other small mammals  
550 picking up their fleas [12]. So the behaviour of rodent species may be also an  
551 important factor explaining the presence of atypical *Bartonella* strains in hosts and  
552 vectors.

553 Among rodent communities, *Bartonella* strains seem to benefit of the  
554 behaviour of hosts as well as the competence of fleas to infest several hosts for  
555 being co-present at a given moment and diversifying by recombination. This idea was  
556 supported by the comparison of genome sequences of two closely related *Bartonella*  
557 species (i.e. the human-specific *B. quintana* and the cat-specific *B. henselae*), but  
558 ecologically different, suggesting that the utilisation of host-restricted vectors is  
559 associated with accelerated rates of genome degradation and may explain why  
560 human pathogens transmitted by specialist vectors are outnumbered compared to  
561 zoonotic agents, which use vectors with broader range of hosts [113], such as  
562 *Bartonella* rodent-associated species.

563 The investigation of *Bartonella* diversity in vector communities infesting rodent  
564 populations and their competence towards the transmission of *Bartonella* strains  
565 associated with rodents may help to elucidate the strategies used by bartonellae  
566 during their life cycle to infect wild rodents.

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569 **V) Interactions between *Bartonella* species and other pathogens**

570

571 As in all ecological communities, other microorganisms infecting rodent  
572 populations can potentially interact positively or negatively with *Bartonella* species,  
573 mostly by modulating the host immune system or by sharing the same resource [1,  
574 114].

575 During surveillance for several tick-borne pathogens (*Borrelia burgdorferi*,  
576 *Babesia microti* and *Ehrlichia* spp.) in the upper Midwest of US, Hofmeister et al. [89]  
577 found *B. vinsonii* subsp. *arupensis* in the blood of white-footed mice (*Peromyscus*  
578 *leucopus*). Surprisingly, they observed that *B. vinsonii* subsp. *arupensis* was  
579 exclusively found in conjunction with *Borrelia burgdorferi* and *Babesia microti* in the  
580 sampled population, hypothesizing that co-infection with these agents may be  
581 relatively frequent in wild mice [89].

582 Thereafter, a 3-year longitudinal study of several haemoparasites in bank  
583 voles (*Myodes glareolus*) conducted in Poland revealed that positive interactions  
584 occur between *B. grahamii* and the bacteria *Mycoplasma* sp. and between  
585 *B. grahamii* and the haemoflagellate *Trypanosoma evotomys* [115]. More recent  
586 studies investigating large natural population of field voles (*Microtus agrestis*) in UK  
587 demonstrated that the presence of cowpox virus increased vole susceptibility to  
588 *B. taylorii* infection as well as the length of bacteraemia [116]. In contrast, *Babesia*  
589 *microti* and *Anaplasma phagocytophylum* had a negative effect on *B. taylorii* infection  
590 probably due to an immune mediated response (cross immunity) and/or a  
591 competition for resources [116-117]. Recently, we reported the coexistence of  
592 *Bartonella* species and *Borrelia afzelii* in a natural population of bank voles (*Myodes*  
593 *glareolus*) sampled across a small area in a French suburban forest. But, based on  
594 statistical inference, we did not find any interaction between both bacteria [85].

595 However, much more known and not yet discovered bacteria, viruses and  
596 protozoan can infect rodents, such as Bunyaviridae or *Toxoplasma* sp., and can act  
597 as modulators for *Bartonella* infection in rodents. Many of these microorganisms are  
598 also transmitted through arthropod vectors. For example, several *B. elizabethae*-like  
599 species have been detected in *Xenopsylla cheopis* infesting rat species [9], which  
600 can also transmit *Yersinia pestis* the agent of plague [118]. So *Bartonella* may also

601 interact with other microorganisms within vectors and may be in competition with  
602 them.

603 Further unbiased global studies highlighting specific interactions with statistical  
604 methods should afford a better understanding of interactions between *Bartonella*  
605 species and other microorganisms living in community within their mammal or  
606 arthropod hosts.

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608

609 **VI) Conclusions**

610 During the last two decades, with the evolution of molecular techniques, an  
611 increasing number of *Bartonella* species have been identified to infect wild rodents in  
612 all continents investigated. These bacteria seem to be ubiquitous in natural  
613 populations of rodent worldwide, exhibiting a huge diversity. We have provided here,  
614 based on the latest knowledge on rodent adapted *Bartonella* species, some  
615 evidences that these bacteria are structured according to the localisation of their  
616 rodent hosts. Furthermore, *Bartonella* species, either at the species or the genotype  
617 level, are clearly highly adapted to their rodent host and arthropod vectors have a key  
618 role in this adaptation. However, knowledge of the competence of vectors, the mode  
619 of transmission, and the bacterial factors involved in arthropod infection and  
620 transmission is still limited.

621 Dynamic and evolution of *Bartonella* species in rodent populations are  
622 subjected to several components, including the rodent host specificity, the  
623 competence of the arthropod vectors, the environment in which the hosts and vectors  
624 evolved, the behaviour of the hosts, and the presence of other microorganisms within  
625 hosts (mammals and vectors). Although some studies have undertaken to investigate  
626 these ecological factors separately, there is a need to take into account all these  
627 aspects when studying *Bartonella* diversity in natural populations.

628 The comparison of the genomes of *B. tribocorum* and *B. grahamii*, associated  
629 to rodents, and that of *Bartonella* species inferred to human and cat revealed that  
630 *Bartonella* species adapted to rodents have a higher amount of host-adaptability  
631 coding genes than the remaining species [107]. In the near future, comparisons of  
632 whole genome sequences should provide novel insights on subtle differences  
633 between molecular mechanisms of closely related *Bartonella* species dictating their  
634 host-vector association.

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639

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969

970 **Table 1.**

971 List of the validated *Bartonella* species associated to rodents, their hosts, potential  
 972 vectors and their medical relevance

Species	Distribution	Host range	Main vector	Evidence of human infection
<i>B. acomydis</i>	Japan	<i>Acomys russatus</i>	?	
<i>B. birtlesii</i>	Europe	<i>Apodemus</i> spp. <i>Myodes glareolus</i>	?	
<i>B. callosciuri</i>	Japan	<i>Callosciurus notatus</i>	?	
<i>B. coopersplainsensis</i>	Asia/Australia	<i>Rattus</i> spp.	<i>Stephanocircus pectinipes</i>	
<i>B. doshiae</i>	Europe/North America	<i>Apodemus</i> spp. <i>Myodes glareolus</i> <i>Microtus agrestis</i> <i>Sigmodon hispidus</i>	<i>Polygenis gwyni</i>	
<i>B. elizabethae</i>	worldwide	<i>Rattus</i> spp. <i>Bandicota</i> spp. <i>Mus</i> spp. <i>Tatera leucogaster</i> <i>Meriones shawii</i> <i>Saccostomus campestris</i> <i>Arvicanthis</i> spp. <i>Praomys</i> spp. <i>Grammomys</i> spp. <i>Aethomys namaquensis</i>	<i>Xenopsylla cheopis</i> <i>Xenopsylla ramesis</i> <i>Rhipicephalus microplus</i>	yes
<i>B. grahamii</i>	Northern Hemisphere	<i>Apodemus</i> spp. <i>Myodes</i> spp. <i>Microtus</i> spp. <i>Mus</i> spp. <i>Rattus</i> spp.	<i>Ctenophthalmus nobilis</i> <i>nobilis</i>	yes
<i>B. jaculi</i>	Japan/Egypt?	<i>Jaculus orientalis</i>	?	
<i>B. japonica</i>	Japan	<i>Apodemus argentus</i>	?	
<i>B. pachyuromydis</i>	Japan	<i>Pachyuromys duprasi</i>	?	
<i>B. phoceensis</i>	Asia/Europe	<i>Rattus</i> spp.	<i>Polyplax spinulosa</i>	
<i>B. queenslandensis</i>	Asia/North America/Australia/Africa	<i>Rattus</i> spp. <i>Bandicota</i> spp. <i>Suncus murinus</i> <i>Mus</i> spp. <i>Arvicanthis</i> spp. <i>Praomys</i> spp. <i>Grammomys</i> spp.	<i>Xenopsylla cheopis</i>	
<i>B. rattaustaliani</i>	Australia	<i>Melomys</i> spp. <i>Rattus</i> spp.	<i>Stephanocircus dasyuri</i>	

<b>B. rattimassiliensis</b>	Asia/Europe	<i>Rattus</i> spp. <i>Bandicota</i> spp. <i>Suncus murinus</i> <i>Mus</i> spp.	<i>Polyplax spinulosa</i> <i>Haemophysalis longicornis</i> <i>Hoplopleura pacifica</i>	
<b>B. rochalimae</b>	Northern Hemisphere	<i>Rattus</i> spp. <i>Myodes glareolus</i> <i>Apodemus</i> spp.	<i>Xenopsylla cheopis</i>	yes
<b>B. silvatica</b>	Japan	<i>Apodemus speciosus</i>	?	
<b>B. tamiae</b>	Thailand	?	<i>Haemophysalis</i> spp. <i>Leptotrombidium</i> spp.	yes
<b>B. taylorii</b>	Europe/Asia	<i>Apodemus</i> spp. <i>Myodes</i> spp. <i>Microtus</i> spp.	<i>Ctenophthalmus nobilis nobilis</i>	
<b>B. tribocorum</b>	worldwide	<i>Rattus</i> spp. <i>Bandicota</i> spp. <i>Mus</i> spp. <i>Aethomys namaquensis</i> <i>Meriones shawii</i> <i>Arvicanthis</i> spp. <i>Praomys</i> spp. <i>Grammomys</i> spp.	<i>Polyplax spinulosa</i> <i>Xenopsylla cheopis</i>	yes
<b>B. vinsonii subsp. arupensis</b>	North America	<i>Peromyscus</i> spp. <i>Onychomys</i> spp.	<i>Oropsylla hirsute</i> <i>Aetheca wagneri</i> <i>Orchopeas leucopus</i>	yes
<b>B. vinsonii subsp. berkhoffii</b>	North America	<i>Sigmodon hispidus</i>	<i>Polygenis gwyni</i>	
<b>B. vinsonii subsp. vinsonii</b>	North America	<i>Neotoma</i> spp. <i>Myodes gapperi</i> <i>Microtus pennsylvanicus</i>	?	
<b>B. washoensis</b>	Northern Hemisphere	<i>Tamias</i> spp. <i>Spermophilus</i> spp. <i>Cynomys</i> spp.	<i>Oropsylla hirsute</i> <i>Aetheca wagneri</i> <i>Orchopeas leucopus</i>	yes

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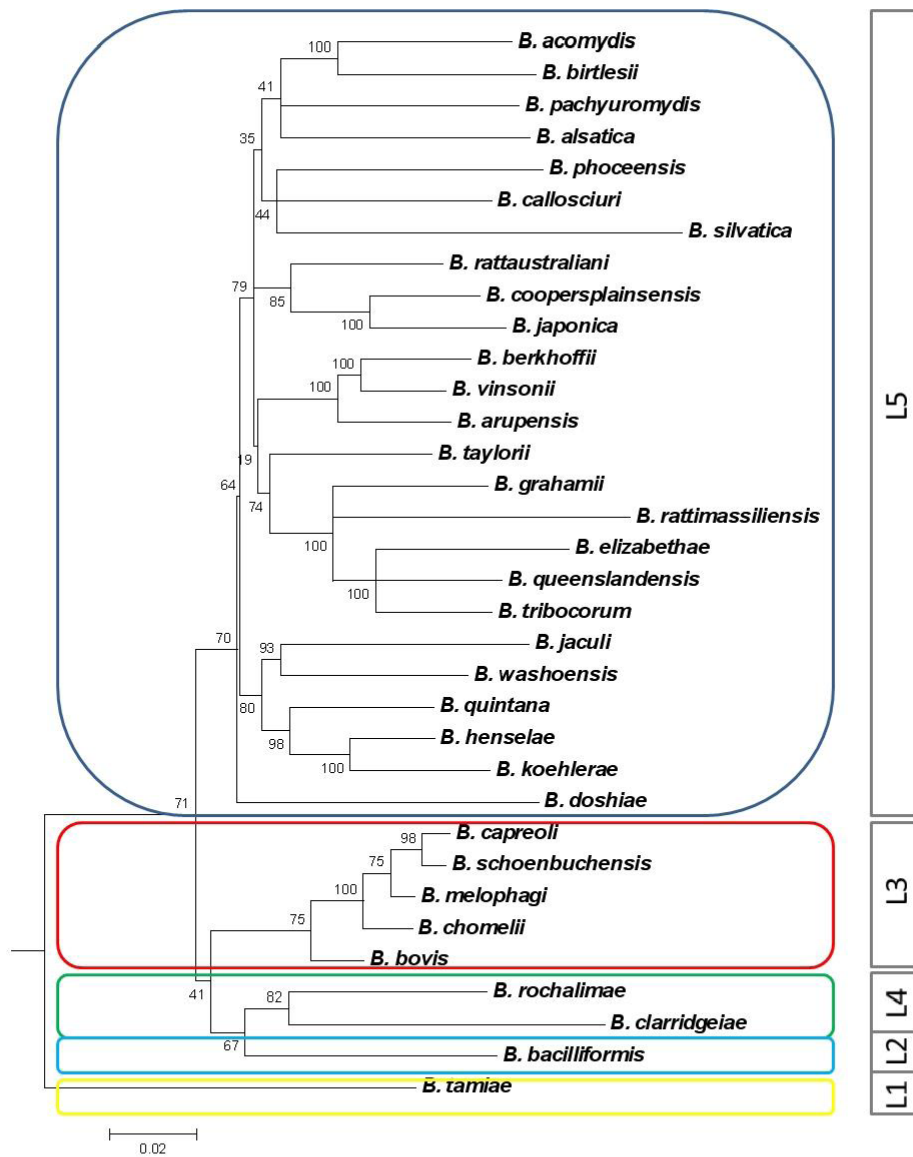
975 **Figure legend**

976

977 **Figure 1.**

978 **Phylogenetic tree of the 32 validated *Bartonella* taxa using the concatenated**  
979 **sequences of 6 genes (16SrRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB*).** The  
980 phylogenetic tree was inferred by using the Maximum Likelihood method based on  
981 the Tamura-Nei model. Bootstrap supports are indicated at the nodes. Phylogenetic  
982 analyses were conducted in MEGA5.

983



984  
985 **Figure 1.**

## **Chapitre II.**

**Diversité et Recombinaison au sein de souches de *Bartonella* colonisant une communauté de rongeurs sylvatiques : vers une meilleure compréhension de la spécificité d'hôte**



## Chapitre II.

# Diversité et Recombinaison au sein de souches de *Bartonella* colonisant une communauté de rongeurs sylvatiques : vers une meilleure compréhension de la spécificité d'hôte (Article 1)

### Introduction

L'interaction entre une bactérie intracellulaire et son hôte fait intervenir des facteurs de virulence de la bactérie qui se lient spécifiquement à des récepteurs présentés à la surface des cellules de l'hôte. Au sein des populations naturelles, la diversité des facteurs de virulence et des récepteurs régit le spectre d'hôte que les bactéries sont capables d'infecter, et définit alors la spécificité d'hôte. La spécificité d'hôte est une caractéristique intrinsèque de nombreuses bactéries pathogènes [22].

Le genre *Bartonella* apparait comme un excellent modèle pour l'étude de la spécificité d'hôte. En effet, chaque espèce de *Bartonella* est adaptée à une ou quelques espèces de mammifère, au sein desquelles la bactérie induit une bactériémie intra-érythrocytaire persistante [2]. Les bartonellae sont transmises par des arthropodes hématophages à leurs hôtes dans lesquels elles colonisent des cellules endothéliales vasculaires en tant que niche primaire. Puis, par vagues successives, les bactéries sont disséminées dans le sang où elles adhèrent et envahissent les érythrocytes. L'invasion des cellules endothéliales et des érythrocytes par les bactéries du genre *Bartonella* dépend de systèmes de sécrétion de type IV (SST4), codés par plusieurs opérons (*trw*, *virB/virD* et *vbh*) compris au sein d'îlots de pathogénicité [2, 23].

Alors que certaines espèces de *Bartonella* sont fortement associées à leur hôte réservoir (telles que *B. henselae* et le chat ou bien *B. bacilliformis* et l'homme), les preuves d'associations entre les bartonellae et les espèces de rongeur varient selon les études et selon la localisation des espèces de rongeurs. En Europe, de nombreuses études ont montré que *B. taylorii*, *B. grahamii* et *B. doshiae* infectent fréquemment plusieurs espèces de rongeurs au même endroit [24-26], évoquant ainsi un manque de spécificité de ces bartonellae pour leurs hôtes mammifères. Cependant, une équipe polonaise a rapporté une forte spécificité d'hôte entre la plupart des génotypes de *Bartonella* (*B. taylorii*, *B. grahamii* et *B. birtlesii*) et les

espèces de rongeurs (*Apodemus flavicollis* et *Myodes glareolus*) à partir desquelles ils ont été isolés [12]. Récemment, la comparaison des génomes complets de 2 espèces de *Bartonella* associées aux rongeurs (*B. tribocorum* et *B. grahamii*) avec les génomes d'espèces inféodées au chat et à l'homme a révélé que les souches hébergées par les rongeurs possèdent une plus grande quantité de gènes codant pour des facteurs de virulence que les autres espèces [27]. D'une manière étonnante, les gènes de virulence de *B. grahamii* sont empaquetés dans des particules de bactériophage permettant aux souches de *B. grahamii* d'échanger du matériel génétique rapidement, ce qui faciliterait les changements d'hôte et expliquerait le manque de spécificité observé auparavant [27].

L'objectif de cet article a été de mieux comprendre l'adaptation des espèces de *Bartonella* à leurs hôtes rongeurs, en isolant et caractérisant des souches de *Bartonella* colonisant une communauté de rongeurs sylvatiques dans une forêt suburbaine française. Nous avons alors établi une phylogénie robuste afin de (i) décrire la diversité des souches circulant au sein de cette communauté de rongeurs ; (ii) déterminer le niveau de spécificité de chaque souche vis-à-vis des espèces de rongeurs ; et (iii) détecter et identifier les transferts horizontaux d'ADN entre les souches de *Bartonella* dans le but de déterminer si elles peuvent co-infecter le même hôte (mammifère ou vecteur). Nous avons enfin examiné la diversité d'un gène de virulence codant pour la protéine VirB5 qui est l'une des composantes du pilus du SST4 VirB/D4 impliquée dans l'invasion des cellules endothéliales, dans le but d'observer une éventuelle association avec l'hôte.

1 **To be submitted to Molecular Ecology**

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6 ***Bartonella* strain diversity dictates rodent host specificity**

7

8

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26 Key words: Bacteria, coevolution, Ecological genetics, Host parasite interaction,  
27 molecular evolution

28 Running title: Host specificity of *Bartonella*

29 **Abstract**

30 Host-specificity is an intrinsic feature of many bacterial pathogens, resulting from a  
31 long history of co-adaptation between bacteria and their hosts. Alpha-proteobacteria  
32 belonging to the genus *Bartonella* infect the erythrocytes of a wide range of mammal  
33 orders, including rodents. To evaluate the specificity of *Bartonella* strains with their  
34 rodent hosts, we performed genetic analysis of *Bartonella* colonizing a rodent  
35 community composed of bank voles (*Myodes glareolus*), wood mice (*Apodemus*  
36 *sylvaticus*) and field voles (*Microtus agrestis*) trapped in a French suburban forest.

37       Following the analysis of 550 rodents, we detected 63 distinct genotypes  
38 related to *B. taylorii*, *B. grahamii*, *B. doshiae* and a new *B. rochalimae*-like species.  
39 Investigating the most highly represented species, we showed that *B. taylorii* strain  
40 diversity was markedly higher than that of *B. grahamii*, suggesting a possible severe  
41 bottleneck for the latter species. The majority of recovered genotypes presented a  
42 strong association with either bank voles or wood mice, with the exception of three  
43 *B. taylorii* genotypes which had a broader host range. Despite the physical barriers  
44 created by host specificity, we observed lateral gene transfer between *Bartonella*  
45 genotypes associated with wood mice and *Bartonella* adapted to bank voles,  
46 suggesting that those genotypes co-habit within their arthropod host during their life  
47 cycle.

48

49 **Introduction**

50

51 Bacterial strain diversification within their natural host populations can restrict  
52 the potential future range of hosts that are susceptible to infection. As a result of  
53 adaptive evolution in their principal host, bacteria have a typically limited host range  
54 that they can successfully infect. At the molecular level, such host-parasite  
55 adaptation is reflected by specific receptor-ligand interactions between bacterial  
56 factors and their target host factors (Finlay& Cossart 1997). The resulting host  
57 specificity is an inherent feature of many bacterial pathogens, including species of  
58 the genus *Bartonella*. Although largely unexplored, the *Bartonella* species represents  
59 an interesting model for investigating the patterns and processes of host specificity  
60 (Vayssier-Taussat *et al.* 2009).

61 *Bartonella* species infect mammalian reservoirs, including rodents, in which  
62 they induce an asymptomatic long lasting intra-erythrocytic bacteremia(Deng *et al.*  
63 2012). To date, more than 30 species and subspecies of *Bartonella* have been  
64 partially or completely characterized. Thirteen of which are recognized as emerging  
65 zoonotic pathogens, causing life-threatening infections in both animal and human  
66 populations. *Bartonella* species are transmitted to their mammalian hosts via the bite  
67 of blood feeding arthropods. They then colonize vascular endothelial cells, from  
68 where they are seeded into the bloodstream before finally adhering to and invading  
69 erythrocytes (Dehio 2004; Deng *et al.* 2012). Infection is driven by two main  
70 pathogenic factors belonging to the type IV secretion systems (T4SS): the  
71 VirB/VirD4, involved in primary niche invasion and adaptation to the mammalian host,  
72 and the Trw T4SS, involved in erythrocyte invasion.

73 As a result of hypothesized adaptive radiation (Engel *et al.* 2011), each of the  
74 30 *Bartonella* species or subspecies only infects one, or a few closely related  
75 mammalian host reservoirs (Deng *et al.* 2012; Engel& Dehio 2009). We have recently  
76 demonstrated that bartonellae host specificity is driven by their unique ability to  
77 adhere and infect erythrocytes from their natural host(s) via the Trw T4SS (Vayssier-  
78 Taussat *et al.* 2010). Equally important for *Bartonella* species host adaptation is the  
79 VirB T4SS machinery, which translocates a cocktail of related effector proteins into  
80 primary niche host cells, where they modulate various processes, resulting in the  
81 ability to adapt to a wide range of different hosts (Engel *et al.* 2011; Saenz *et al.*  
82 2007).

83           While some species are highly specific for their host (i.e., *B. henselae* for the  
84 cat or *B. bacilliformis* for humans), other specific associations do not seem so clear-  
85 cut, especially for rodent-associated species. In addition the association between  
86 some *Bartonella* species and their rodent hosts remains a matter of controversy.  
87 Indeed, while *B. vinsonii* subsp. *arupensis* and *B. washoensis* have only been found  
88 to infect *Peromyscus* mice and ground squirrels respectively (Bai *et al.* 2011; Inoue  
89 *et al.* 2011; Jardine *et al.* 2005; Kosoy *et al.* 2003), *B. elizabethae* has been found to  
90 infect both *Rattus* rats in Southern China (Ying *et al.* 2002) and *Bandicota* rats in  
91 Thailand . In Europe, *B. taylorii*, *B. grahamii* and *B. doshiae* infected several  
92 sympatric woodland rodents at a single site (Berglund *et al.* 2010a; Birtles *et al.*  
93 2001; Buffet *et al.* 2012; Holmberg *et al.* 2003), while a longitudinal study realized in  
94 Poland reported a strong host association between the majority of *Bartonella* species  
95 and their rodents (*Apodemus* mice and *Myodes voles*) (Paziewska *et al.* 2012a).  
96 Recently, the complete genome sequences of two rodent-carried *Bartonella* species  
97 (i.e. *B. tribocorum* and *B. grahamii* strains) were compared to *Bartonella* species from  
98 humans and cats, revealing that the rodent-associated species carried a higher  
99 number of T4SS host-adaptability factor encoding genes (Berglund *et al.* 2009).  
100 Interestingly, these host-adaptability genes are packaged into bacteriophage  
101 particles, resulting in an original method of gene exchange between *B. grahamii*  
102 strains promoting rapid diversification. This could therefore facilitate host shifting,  
103 which may explain the observed lack of host specificity of some species or strains  
104 (Berglund *et al.* 2009).

105           In order to improve our understanding of how *Bartonella* species are  
106 associated with their mammalian hosts, it is important to accurately describe  
107 *Bartonella* species diversity within their natural host. Previous multi-locus sequence  
108 typing methods (MLST) of *B. henselae* strains isolated from humans and cats  
109 sampled across several continents revealed 14 genotypes, but did not disclose an  
110 obvious host association pattern, except for one genotype restricted to European  
111 cats (Arvand *et al.* 2007). However, a more recent MLST scheme based on eight loci  
112 indicated that the majority of human isolates are rare genotypes differing from those  
113 of cats (Chaloner *et al.* 2011). Moreover, a variable number tandem repeat analysis  
114 performed on 178 *B. henselae* strains resulted in 99 profiles separated into two  
115 groups, where all human isolates were clustered within the same group  
116 (Bouchouicha *et al.* 2009). All typing schemes applied to *B. quintana* strains, strictly

117 human specific, indicated very low levels of diversity (Arvand *et al.* 2010; Foucault *et*  
118 *al.* 2005). In relation to rodent-adapted *Bartonella* species, the only published study  
119 utilized multi-locus sequence analysis (MLSA) of *B. grahamii* strains sampled  
120 worldwide, and demonstrated strict host specificity in Asia and North America, and  
121 low host specificity in Europe (Berglund *et al.* 2010b; Inoue *et al.* 2009).

122 Typically, intra-cellular bacteria have lower recombination rates, probably due  
123 to their relative niche isolation (Vos& Didelot 2009) and indeed, the *Bartonella* genus  
124 has one of the lowest recombination rates among bacteria (Vos& Didelot 2009). This  
125 might be due to its intracellular location but also to its restricted host range.  
126 Interestingly, within the *Bartonella* genus, strains circulating within rodents showed  
127 more frequent recombination events compared to human and cat adapted species,  
128 suggesting a broader host range for rodent adapted species (Berglund *et al.* 2010b;  
129 Paziewska *et al.* 2011).

130 To learn more about the adaptation of *Bartonella* species to their wild animal  
131 reservoirs, we conducted a field study in which *Bartonella* strains were isolated from  
132 a rodent community living within a suburban forest. To determine the level of strain  
133 specificity for rodent species, we performed robust genetic diversity analysis on the  
134 recovered *Bartonella* strains, based on a Multi-locus Sequence Analysis (MLSA)  
135 using six housekeeping genes. Recombination events between strains were  
136 analyzed to evaluate whether these strains have had the opportunity to co-habit.  
137 Finally, due to the important role of the T4SS VirB/D4 in *Bartonella* species host  
138 adaptability, we also investigated whether VirB5 diversity revealed a possible host  
139 association. This study allowed us to understand how rodent-adapted *Bartonella*  
140 species co-habit, exchange genes and interact within this rodent community.



141

## 142 **Materials and Methods**

143

### 144 *Study site and animal processing*

145 The study was conducted between 2007 and 2008 in the Forest of Sénart  
146 (3,200 ha, 02°29'E, 48°40'N), which is located in a dense urbanized area 22 km  
147 south-east of Paris (Buffet *et al.* 2012). This forest is visited by approximately three  
148 million visitors per year (Marsot *et al.* 2011). Micromammals were trapped within a  
149 baited live-trapping grid system, arranged in a staggered network consisting of 104  
150 Sherman<sup>®</sup> traps covering the park of La Faisanderie (12 ha) (Chapuis *et al.* 2011;  
151 Marmet *et al.* 2009) situated in the centrum of the forest. The design was checked  
152 four times a day over five consecutive days at monthly interval. Captures of nocturnal  
153 rodents were only performed during one month (April 2008) from the addition of four  
154 rectangular grids, each made by 60 Sherman traps covering approximately 1.6 ha,  
155 baited and checked three times a day then kept opened for the night. This was  
156 performed over four consecutive days and nights (Buffet *et al.* 2012). Rodent species  
157 (*Apodemus sylvaticus*, *Myodes glareolus* and *Microtus agrestis*) were determined  
158 according to morphological criteria provided by Quéré & Le Loarn (Le Louarn & Quéré  
159 2003).

160 Over the entire study period, 550 rodents belonging to three species were  
161 captured. The bank vole *Myodes glareolus* (n = 471) dominated the diurnal  
162 community, and the field vole *Microtus agrestis* (n = 9) was rare. Nocturnal species  
163 only comprised wood mice *Apodemus sylvaticus* (n = 70). Rodents were then  
164 sacrificed and their spleens removed under sterile conditions and stored at -80°C until  
165 they were to be used for *Bartonella* culture and/or DNA PCR detection.

166

### 167 *Bacterial isolates and culture conditions*

168 Bacterial isolation was performed by grinding a piece of spleen in 500 µl of F-  
169 12 nutrient mixture medium (Invitrogen), and then plating 250 µl of the mixture onto  
170 Columbia agar containing 5% defibrinated sheep's blood (CBA), which was then  
171 incubated at 35°C in a humidified atmosphere with 5 % CO<sub>2</sub> from 5 to 30 days.

172

### 173 *PCR amplification and sequencing of protein-coding genes*

174 DNA was extracted from rodent spleen samples as previously described  
175 (Buffet *et al.* 2012), and bacterial isolates was heated in 200 µl suspension at 95°C  
176 for 10 min, followed by a 5 min centrifugation step of 6,000 g at 4°C. The supernatant  
177 was then collected and used for PCR. The presence of *Bartonella* DNA was  
178 determined by using a portion of the *gltA* gene as previously described (Buffet *et al.*  
179 2012). For positive samples, five other protein-coding gene sequences were  
180 amplified for MLSA analysis: cell division protein gene (*ftsZ*), NADH dehydrogenase  
181 gamma-subunit gene (*nuoG*), 60kDa heat-shock protein gene (*groEL*), riboflavin  
182 synthase gene (*ribC*), and RNA polymerase beta-subunit gene (*rpoB*) using primers  
183 previously described (Colborn *et al.* 2010; Paziewska *et al.* 2011; Renesto *et al.*  
184 2001; Zeaiter *et al.* 2002a; Zeaiter *et al.* 2002b).

185 For *virB5* analysis, the 447-522 bp region was amplified and sequenced from 62  
186 DNA samples and 31 isolates representing the diversity of *Bartonella* strains  
187 recovered in this study. To amplify the complete *virB5* gene, primers were designed  
188 as follows: the forward primer virB5F-B4: 5'-GCA-GAA-CTY-AAY-TTA-CGK-GG-3'  
189 was complementary to the 3' end of the *virB4* gene (accession numbers,  
190 NC\_012846, NC\_010161, NC\_014932, NC\_005956), and the reverse primer  
191 virB5R3-B6: 5'-GCA-TTY-GTT-GCC-ATT-GTT-GTC-AC-3' complemented the 5' end  
192 of the *virB6* gene (accession numbers, NC\_012846, NC\_010161, NC\_014932,  
193 NC\_005956), resulting in complete coverage of the *virB5* gene.

194 PCR amplification was performed in a final volume of 25 µl containing 0.5 µM  
195 of each primer, 3% (vol/vol) of dimethyl sulfoxide, 200 µM of each deoxynucleotide  
196 triphosphate, 1x Phusion™ GC buffer, 0.4 U of Phusion™ DNA polymerase  
197 (Finnzymes) and 20-50 ng of genomic DNA. The PCR amplification conditions were  
198 as follows: an initial cycle of 95°C for 5 min; 37 amplification cycles, each consisting  
199 of 95°C for 30 s, 54°C (*gltA*), 55°C (*ftsZ*), 54°C (*groEL*), 53°C (*rpoB*), 48°C (*ribC*),  
200 53°C (*nuoG*) and 48°C (*virB5*) for 30 s, followed by an elongation step of 72°C for 1  
201 min; and a final incubation at 72°C for 10 min. Amp lification products were analyzed  
202 by electrophoresis on 2.5% agarose gels with 0.1 mg/ml of ethidium bromide and  
203 verified under UV light. PCR products were then purified and sequenced on both  
204 strands by Eurofins MWG Operon (Germany).

205 To validate all amplified sequences, we repeated DNA extraction, amplification  
206 and sequencing for each spleen sample and isolate.

207

208 *Phylogenetic analysis and recombination tests*

209 Sequences were edited and aligned using Geneious™ v5.6.4 software.  
210 Nucleotide diversity indices and the polymorphic level were calculated using DNAsp  
211 v5.10 (Librado& Rozas 2009). Neighbor-joining tree analysis for each individual gene  
212 was performed using MEGA v5 (Tamura *et al.* 2011). For the MLSA approach,  
213 individual gene sequences were concatenated. The best model of nucleotide  
214 substitution was determined with jModelTest (Posada 2008). A phylogeny was  
215 constructed based on the concatenates by using the maximum-likelihood method  
216 implemented in PhyML software (Guindon& Gascuel 2003). Missing genes were  
217 considered as deletions. *Bartonella* reference strain sequences are listed in Table  
218 S1.

219 To build the phylogeny of *virB5* sequences, the best model of nucleotide  
220 substitution was calculated with jModelTest, and the phylogeny constructed using the  
221 maximum-likelihood method with MEGA v5.

222 A phylogenetic tree was also inferred using ClonalFrame v1.1 (Didelot&  
223 Falush 2007) with the number of MCMC iterations set to 200,000, following a burn-in  
224 period of 100,000 iterations. ClonalFrame is a Bayesian inference method which  
225 jointly reconstructs the clonal relationships between the isolates in a sample, as well  
226 as the location of recombination events that have disrupted the clonal signal.

227 Allelic profiles were identified for each individual *Bartonella* strain, and an  
228 allele number was assigned to every distinct sequence variant for each of the six loci,  
229 using the BioNumerics v6.5 software (Applied-Maths, Sint Maartens-Latem,  
230 Belgium). To test for linkage disequilibrium between alleles of the six analyzed loci,  
231 the Index of association (Ia) between alleles (Smith *et al.* 1993) was calculated using  
232 the START program (<http://pubmlst.org>).

233 Recombination test calculations were performed using RDP3 (Martin *et al.*  
234 2010). The Neighbor-Net implemented in the software SplitsTree 4.12 (Huson&  
235 Bryant 2006) with 1,000 bootstrap replicates was used to create the phylogenic  
236 network for the concatenated sequences. Furthermore, we used the pairwise  
237 homoplasmy index (PHI) (Bruen *et al.* 2006) in SplitsTree 4.0 in order to test the role of  
238 past recombination events in generating allelic variation. To estimate the relative  
239 contribution of recombination and mutation events among *Bartonella* genotypes, we  
240 performed two independent runs of the analysis tool ClonalFrame (Didelot& Falush  
241 2007), each consisting of 200,000 MCMC iterations, discarding the first half as burn-

242 in. We used the linkage model in Structure v2.3 (Hubisz *et al.* 2009) to identify  
243 populations with distinct allele frequencies present in our data and estimate genetic  
244 exchange among these populations. We performed five runs, each using a different  
245 value of between two and eight for the number of populations ( $K$ ), each with 100,000  
246 MCMC iterations, following a burn-in period of 50,000 iterations. These tests indicated  
247 that the model probability was highest at  $K = 4$ .

248

#### 249 *Nucleotide sequences*

250 Sequences of the six protein-coding genes and the *virB5* gene were deposited  
251 in GenBank/EMBL/DDBJ databases under the accession numbers JX846090 to  
252 JX846493 (Table 1).

253

254

## 255 **Results**

256

### 257 *Phylogenetic relationships of Bartonella genotypes, delineation of genetic clusters*

258 To characterize the diversity of *Bartonella* circulating in the rodent community,  
259 we first performed a phylogenetic analysis based on the alignment of the  
260 concatenated sequences of the six housekeeping genes, using the 212 *Bartonella*  
261 *gltA*-positive DNA extracts and the 43 *Bartonella* strains. The phylogenetic tree (Fig.  
262 1a) was generated using maximum likelihood (PhyML) with a K81 substitution model  
263 (estimated using jModelTest). Only one representative strain of each genotype was  
264 reported on the phylogenetic tree. This analysis revealed the phylogenetic  
265 relationships of the 63 unique genotypes with three known *Bartonella* species:  
266 *B. taylorii*, *B. doshiae*, and *B. grahamii*; and a misidentified genotype (A296, cluster  
267 H) closely related to *B. rochalimae* and *B. clarridgeiae* (8.2 and 9.3% nucleotide  
268 divergence respectively). *B. grahamii* (cluster G) did not exhibit an obvious internal  
269 phylogenetic structure, except for one genotype (A621) recovered from a single bank  
270 vole, which was genetically diverged from all the other *B. grahamii* genotypes by 2.4  
271 to 2.9%. In contrast, within the *B. taylorii* species, the phylogenetic tree showed a  
272 clear demarcation of five clades (A, B, C, D and E) with high bootstrap support (81 –  
273 100%). Each clade formed distinct compact clusters separated by genetic distances  
274 ranging from 1.3 to 3.5%, according to demarcation parameters within a bacterial  
275 species (Palys *et al.* 1997). Two genotypes were associated with the species  
276 *B. doshiae* (cluster F), of which one (A340) was more closely related to the R18  
277 reference strain with a genetic distance of 0.7%, and another (A538) more distant  
278 which diverged by a distance of 4.5%.

279

### 280 *Host specificity of Bartonella genotypes*

281 When considering the specific association between *Bartonella* species and  
282 their rodent hosts, all *B. grahamii*, *B. doshiae* and the *B. rochalimae* related species  
283 genotypes were only recovered from bank voles. At the species level, *B. taylorii*  
284 strains were recovered from field voles, bank voles and wood mice with an apparent  
285 host preference for bank voles (93,3 % of *B. taylorii* strains were isolated from bank  
286 voles). However, data indicate host specificity at the genotype level with *B. taylorii*  
287 clusters B and C specific to bank voles, and cluster E specific to wood mice. Clusters

10



288 A and D have a larger host range and are detected mainly in bank voles and to a  
289 lesser extent in wood mice (cluster A) or field voles (cluster D) (Table 1).  
290 Interestingly, genotypes infecting wood mice (A140 and A447) from cluster A and  
291 genotypes infecting field voles (A145) from cluster D can also infect bank voles (Fig.  
292 1a).

293

#### 294 *Genetic polymorphism and recombination within rodent adapted Bartonella species*

295 We examined sequence polymorphism of the six housekeeping genes in 72  
296 out of 212 *Bartonella* strains of *B. taylorii* and *B. grahamii*. *B. doshiae* and *B.*  
297 *rochalimae* related species were excluded from the study because of the low number  
298 of strains. Following alignment of the 3448 nucleotides of the six gene fragments, a  
299 total of 793 (23%) polymorphic sites were found, discriminating 41 individual alleles  
300 (Table 2). The number of polymorphic sites on a given locus varied from 56 (17.2%)  
301 for *nuoG* to 217 (26.72%) for *groEL*. The number of individual alleles for each of the  
302 six protein-coding genes ranged from 8 for *ftsZ* to 28 for *groEL*. Most polymorphisms  
303 resulted in synonymous substitutions, and the ratio of non-synonymous to  
304 synonymous substitutions (*dN/dS*) varied from 0.016 (for *nuoG*) to 0.24 (for *ribC*)  
305 (Table 2). These low ratios indicate a combination of purifying selection on amino-  
306 acid variation and a lack, or a very limited contribution, of positive environmental  
307 selection to the sequence variation in the six analyzed loci. These genes are  
308 therefore assumed to be appropriate for a population genetic study.

309 The average nucleotide diversity ( $\pi$ ) within all *Bartonella* genotypes was 5.2%  $\pm$   
310 0.471, ranging from 4.4% to 7.5% per gene. Within the species *B. grahamii*, the  
311 average nucleotide distance was restricted ( $\pi = 0.159\% \pm 0.054$ , Table 2), indicating  
312 that the core genome of *B. grahamii* is relatively homogeneous, whereas *B. taylorii*  
313 ( $\pi = 1.76\% \pm 0.211$ ) was much more diverse. We also noted striking intergene  
314 variation in levels of diversity within *B. taylorii* and *B. grahamii* genotypes: *nuoG*  
315 ( $\pi = 4.2\% \pm 0.458$ ) sequences were found to be more variable than sequences of  
316 *gltA* (2.2%  $\pm 0.255$  on average) within *B. taylorii* species; within *B. grahamii*, *groEL*  
317 sequences (0.3  $\pm 0.077$ ) were more heterogeneous than those of *gltA* (0.077%  $\pm$   
318 0.065), whereas *nuoG* sequences (1 allele, no polymorphic site) were homogeneous.

319 A quantitative analysis of the association between alleles from the six loci, by  
320 calculating the Index of Association (*I<sub>a</sub>*) (Smith *et al.* 1993), estimated the  
321 contribution of recombination to the genetic structure among individuals of a

322 population. Including all the *Bartonella* strains in the analysis, significant linkage  
323 disequilibrium was detected ( $I_a = 1.999 \pm 0.846$ ), as expected for distinct species.  
324 Within *B. taylorii* genotypes,  $I_a$  was calculated as  $1.847 \pm 0.988$ , confirming the  
325 significant linkage disequilibrium between alleles and hence a deviation from a  
326 random-mixing population within this species. In contrast, among *B. grahamii* strains,  
327 with a value of  $I_a$  at  $0.128 \pm 0.761$ , no significant linkage disequilibrium was detected  
328 indicating frequent homologous recombination events.

329

#### 330 *Relationships between host specificity of rodent adapted Bartonella species and* 331 *homologous recombination among clusters*

332 Throughout the entire *Bartonella* population, the inferred recombination to  
333 mutation value (*rim* ratio) calculated with ClonalFrame was estimated at 4.06 ( $CI_{95} =$   
334 [2.28; 6.38]), suggesting that nucleotide changes in housekeeping genes occur more  
335 frequently by recombination than by *de novo* mutation. Among the rodent  
336 community's two most abundant *Bartonella* species, recombination rather than  
337 mutation was found to have played a more important role within *B. taylorii* as  
338 compared to *B. grahamii* ( $rim = 6.81$  and  $3.77$ , respectively). To examine any  
339 possible influences of recombination on the tree topology (Fig. 1a), we inferred a  
340 phylogenetic tree of the complete dataset using ClonalFrame, which takes  
341 recombination into account during tree building. Analysis resulted in a tree with the  
342 same major clades as identified by MLSA (supplementary Fig. S1).

343 We then evaluated whether recombination was detected between clusters by  
344 using several different approaches. Firstly, we individually compared the phylogeny  
345 of each gene and demonstrated that *Bartonella* genotype position varied depending  
346 upon the gene (supplementary Fig. S2). Indeed, individual *ftsZ*, *gltA*, *ribC* and *rpoB*  
347 phylogenies showed strong congruence among clusters, but with some remarkable  
348 discrepancies. For example, as opposed to the concatenate or to five other genes,  
349 *gltA* did not strongly associate all of the genotypes belonging to cluster A. In contrast  
350 for this phylogeny, genotypes of cluster A were separated into two clades, of which  
351 one merged in a short branch with cluster B and C genotypes (supplementary Fig.  
352 S2). This observation could be attributed to the horizontal transfer of the *gltA* gene  
353 from a donor related to cluster A, into an ancestral strain of clusters B and C. The  
354 *rpoB* sequence of the genotype A538 (*B. doshiae*) was identical to cluster A of  
355 *B. taylorii* sequences. Likewise, in *ribC* phylogeny, we identified a different position

12



356 for 10 genotypes among clusters A, C, D (*B. taylorii*), G (*B. grahamii*) and H. For  
357 example, the *ribC* sequences of genotypes A296 (cluster H), A132 and A193 (cluster  
358 D) were almost identical to *B. grahamii* sequences, whereas the *ribC* sequence of  
359 A621 (*B. grahamii*) was identical to that of *B. taylorii* cluster A genotypes  
360 (supplementary Fig. S2). *GroEL* and *nuoG* phylogenies were more discordant in their  
361 phylogenetic placement of genotypes. For the *nuoG* phylogeny, cluster C genotypes  
362 were distributed amongst all clusters, and genotype A296 was associated with  
363 cluster D. The *groEL* phylogeny showed an association of *B. taylorii* and *B. doshiae*  
364 genotypes to the *B. birtlesii* reference strain IBS 325, strongly supported by high  
365 bootstrap (supplementary Fig. S2). These results, which were confirmed by re-  
366 sequencing of the six genes from new DNA extracts, demonstrated the occurrence of  
367 horizontal gene transfer between distantly related *Bartonella* species, as has been  
368 previously described (Paziewska *et al.* 2011).

369 Secondly, in order to detect those more subtle recombination events which  
370 result in detectable footprints of foreign DNA incorporation, nucleotide  
371 polymorphisms within the six genes were analyzed with the Structure software  
372 (Hubisz *et al.* 2009). Structure recognized four clusters (A, D, E and G), and most  
373 strains within the identified clusters were homogeneous in terms of their ancestral  
374 polymorphism source (Fig. 1b). However, as expected, the transfer of gene  
375 sequences from other clusters were visualized by Structure, as is evident from the  
376 mixed color of the corresponding genotype column (Fig. 1b). Moreover, Structure  
377 revealed a number of genotypes that appeared to have a partially mixed origin, such  
378 that a mosaic pattern was seen where recombination of a large contiguous part of a  
379 gene had occurred. For example, a 323 bp segment of *ribC* from genotype A132  
380 (*B. taylorii*, cluster D, recovered from a bank vole) was nearly identical to *B. grahamii*  
381 sequences (cluster G specific to bank voles), while a 293 bp segment of *groEL* was  
382 identical to sequences of *B. taylorii* cluster E genotypes (specific to wood mice).  
383 Furthermore, genotype A550 (*B. taylorii*, cluster D recovered from bank voles)  
384 contained a 313 bp *ribC* segment nearly identical to *B. taylorii* cluster A sequences  
385 (recovered from both bank voles and wood mice). Likewise, genotype A621  
386 (*B. grahamii*, cluster G) exhibited a mixed origin, with *ribC* sequences matching those  
387 in *B. taylorii* clusters A and E (344 and 556 bp respectively). Interestingly, a number  
388 of *B. taylorii* and *B. grahamii* strains originally isolated from bank voles were likely to  
389 have a mixed origin, as sequences were found to be identical to those from *B. taylorii*

390 cluster E, which were exclusively found in wood mice. This sequence mosaicism was  
391 supported by at least three different algorithms in RDP3 and SplitsTree ( $p < 0.05$ ).  
392 Thus, Structure analysis disclosed evidence of recombination events among *B.*  
393 *taylorii* related genotypes, as well as between *B. taylorii* and *B. grahamii* genotypes,  
394 independent of the rodent species from which they were recovered.

395 These independent recombination events were further confirmed by  
396 performing a phylogenetic network analysis using the Neighbor-Net method.  
397 Evidence of extensive homologous recombination within the different genotypes,  
398 including cluster E, was supported by visual inspection of the bushy network  
399 structure with complex parallelogram formation (supplementary Fig. S3).

400

#### 401 *Diversity and allele sharing of virB5 coding sequences among Bartonella clusters*

402 Due to the important role of the VirB/D4 type IV SS in host adaptability of  
403 *Bartonella* species (Engel *et al.* 2011), we investigated the diversity and homologous  
404 recombination events among the pilus component-coding *VirB5* sequences.

405 *VirB5* sequences were amplified and sequenced from 44 bank vole DNA  
406 extracts, two *Bartonella* DNA extracts specific to wood mice (A554 and A286 from  
407 cluster E) and zero from field mice. Overall, the coding sequences of virulence factor  
408 *virB5* showed 12 alleles undergoing a distinctive pattern of evolution. In relation to  
409 the entire *virB5* gene, calculation of the  $dN/dS$  ratio (1.145) disclosed a weak  
410 purifying selection and/or diversifying selection. Differences between *virB5* alleles  
411 were due to mutation, recombination events and the loss/gain of indels (Paziewska *et al.*  
412 2012b). In all, the inferred *VirB5* product ranged from 149 to 174 amino acids (aa)  
413 in length.

414 A phylogenetic tree (Fig. 2) was generated using maximum likelihood with a  
415 GTR substitution model. *VirB5* sequences were clustered into seven clades (from  
416 clusters 1 to 7, in Fig. 2). In contrast to housekeeping genes, phylogenetic analysis of  
417 *virB5* sequences merged those sequences related to *B. taylorii* cluster C (specific to  
418 bank voles) with that of *B. taylorii* cluster D (infecting mainly bank voles and to a  
419 lesser extent, field mice), with 1.1% nucleotide divergence (see cluster 3 in Fig. 2).  
420 This phylogenetic analysis also uncovered a conspicuous pattern of homologous  
421 recombination of short intragenic regions and entire *virB5* sequences among the  
422 clusters, resulting in mosaic-like structures. The *virB5* sequence of genotype A550  
423 (*B. taylorii*, cluster D with MLSA, recovered from bank voles) was identical to cluster

424 A sequences (infecting mainly bank voles, and wood mice to a lesser extent).  
425 Likewise, the *virB5* sequence of genotype A554 (cluster E with MLSA, infecting wood  
426 mice) was merged with those from cluster A. Moreover, the *virB5* sequences of  
427 genotype A612 and A357 (cluster B infecting bank voles) were nearly identical to  
428 those of *B. grahamii* (exclusively infecting bank voles).

429         These results indicated that *virb5* sequences are distributed across the clades  
430 identified with MLSA analysis, clearly demonstrating that *virB5* alleles are not  
431 structured according to rodent host species.

432 **Discussion**

433

434 By studying the diversity of *Bartonella* strains recovered from a rodent  
435 community comprising three main rodent species, we provide evidence of strong  
436 host-specific associations between *Bartonella* genotypes and their hosts.  
437 Interestingly, we also highlight the existence of many recombination events between  
438 different *Bartonella* species and genotypes, demonstrating that even though these  
439 genotypes do not share the same rodent host species, their host range partially  
440 overlaps, providing opportunities for recombination.

441 Until now, the majority of studies describing *Bartonella* populations within  
442 rodent communities used a *gltA* gene sequence to identify and characterize  
443 *Bartonella* genotypes. As shown in this study, many recombination events occur  
444 within this gene. Thus, only using this gene might lead to biases resulting in the false  
445 identification of genotypes (Paziewska *et al.* 2011). To obtain unbiased and more  
446 discriminatory *Bartonella* genotype identification, we used six housekeeping genes,  
447 as well as the host adaptability *VirB5*-encoding gene, enabling a more precise  
448 overview of *Bartonella* strain diversity from distinct rodent species. The present work  
449 unambiguously confirmed the high diversity of *Bartonella* genotypes recovered from  
450 woodland rodents as demonstrated in previous studies (Berglund *et al.* 2010a;  
451 Paziewska *et al.* 2012a; Telfer *et al.* 2007), with 63 different *Bartonella* genotypes  
452 retrieved from 550 animals. These genotypes were separated into three known  
453 *Bartonella* species: *B. taylorii*, *B. grahamii* and *B. doshiae*, commonly found in  
454 Europe, and one genotype related to *B. rochalimae*-like isolates obtained from wood  
455 mice in Sweden and Spain (Gil *et al.* 2010; Holmberg *et al.* 2003). The percentage of  
456 nucleotide divergence between this genotype and *B. rochalimae* is 8.2%, based on  
457 MLSA. As a nucleotide divergence greater than 5% within housekeeping genes is  
458 now considered as equivalent to the 70% DNA-DNA re-association criteria used for  
459 species demarcation (Konstantinidis & Tiedje 2005), *B. rochalimae*-like strains isolated  
460 here and elsewhere in Europe correspond to a new species. Therefore it will be  
461 hereafter referred to as the "*B. rochalimae*-like" new species.

462 To highlight specific associations between *Bartonella* and their mammalian  
463 hosts, our strategy was to study the diversity of *Bartonella* strains isolated from  
464 different sympatric rodent species. Globally, the *B. taylorii* population, identified in  
465 158 individuals (bank voles, field voles and wood mice), was much larger than the

466 *B. grahamii* population, which was only recovered from 28 bank voles. *B. doshiae*  
467 and *B. rochalimae*-like sequences were the least abundant genotypes, recovered  
468 from six and one bank vole(s) respectively. Interestingly, we identified much higher  
469 diversity within the five *B. taylorii* clades (1.76% using MLSA), compared to  
470 *B. grahamii* (0.2%) which were grouped into a single cluster. These large differences  
471 in diversity were also corroborated by the *virB5* phylogeny, which distinguished nine  
472 different alleles for *B. taylorii*, while only one *virB5* sequence was identified for  
473 *B. grahamii*. Similar homogeneity within *B. grahamii* strains has been observed in  
474 both the UK and Sweden (0.1%) (Berglund *et al.* 2010b). These low polymorphism  
475 rates may indicate that *B. grahamii* in Europe experienced a severe bottleneck (i.e., a  
476 reduction in population size) relatively recently, with too little time having elapsed for  
477 polymorphisms to re-accumulate. An alternate hypothesis is that *B. grahamii* has  
478 shifted hosts from bank voles to another sympatric rodent species not sampled in this  
479 study, as suggested for *B. quintana* (Foucault *et al.* 2005). One such host could be  
480 the introduced Siberian chipmunk (*Tamias sibiricus*) which inhabits the studied area  
481 in close relation to bank voles and wood mice (Pisanu *et al.* 2007). The low  
482 polymorphism rate within *B. grahamii* is also consistent with the simultaneous  
483 diversification of *B. taylorii* in multiple lineages, due to a probable rapid population  
484 expansion in order to occupy empty ecological niches following the proposed  
485 *B. grahamii* bottleneck. The bottleneck endured by *B. grahamii* may be also due to its  
486 restriction to a single vector; while the expansion and the diversification of *B. taylorii*  
487 could be explained by its ability to be transmitted by a wider range of vectors.  
488 Systematic surveying of the fleas infesting the bank voles detected three flea  
489 species: *Ctenophthalmus agyrtes impavidus*, *Megabothris turbidus*, and to a lesser  
490 extent *Ctenophthalmus congener* (Pisanu & Chapuis, unpublished data). Further  
491 studies on the prevalence of *Bartonella* genotypes within rodent flea communities, as  
492 well as the competence of these fleas for the *Bartonella* rodent-associated species  
493 transmission, should help us to understand the differences in genetic structure  
494 observed in these two *Bartonella* species.

495 We demonstrated that the *Bartonella* population hosted by bank voles was  
496 more diverse than those retrieved from wood mice or field voles (Table 1). We have  
497 also shown that clusters B and C of *B. taylorii*, *B. grahamii*, *B. doshiae* and “*B.*  
498 *rochalimae*-like” have strict host specificity for bank voles, and cluster E of *B. taylorii*  
499 for wood mice. A broader host range has been identified for some genotypes



500 belonging to clusters A and D (of *B. taylorii*), that can infect both bank voles or wood  
501 mice, or bank voles and field voles respectively. Seasonal host density is known to  
502 drive which *Bartonella* species infects sympatric rodents (Telfer *et al.* 2007).  
503 However, in our study, vole and mouse population abundance, evaluated during the  
504 second year of trapping (2008), remained relatively stable. Our results suggest that  
505 host density plays a minor role in explaining the differences in *Bartonella* infection  
506 between voles and mice. Rather, host susceptibility might have a major impact on the  
507 different patterns of *Bartonella* diversity according to rodent species.

508 For intracellular bacterial species, in particular *Bartonella* species,  
509 recombination rates are generally low. Interestingly, the ratio of recombination to  
510 mutation ( $r/m$ ) across the entire *Bartonella* population was estimated to be 4.06,  
511 which was much higher compared to that of *B. henselae* (0.1) (Vos& Didelot 2009).

512 In the particular case of *B. grahamii*, we obtained the high  $r/m$  ratio of 3.77,  
513 compared to 1.7 for *B. grahamii* strains published in the study by Berglund *et al.*  
514 (Berglund *et al.* 2010b). This difference may be due to the population of *B. grahamii*  
515 isolates sampled. Indeed, the authors performed analysis on *B. grahamii* strains  
516 collected worldwide, whereas we focused our analysis on sympatric strains sampled  
517 from a small area. Interestingly, even though *B. grahamii* strains had a low  
518 polymorphism rate, we did identify recombination events, suggesting that perhaps  
519 the resulting recombinants did not persist within bank voles, due to a possible  
520 inability to adapt to their host. Another remarkable finding was that the contribution of  
521 recombination in generating diversity among *B. taylorii* ( $r/m = 6.81$ ) was almost twice  
522 as high as that seen within *B. grahamii* ( $r/m = 3.77$ ). These results reflect the greater  
523 diversity of *B. taylorii* strains compared to *B. grahamii*, and are also consistent with  
524 the wider host range of *B. taylorii*. The analysis of the complete genome sequence of  
525 a *B. grahamii* strain isolated in Sweden has lead to the discovery of a gene transfer  
526 agent (GTA) that packages random bacterial DNA (Berglund *et al.* 2009). In relation  
527 to our results, the high recombination rates observed among *B. taylorii* strains  
528 strongly suggest that *B. taylorii* may harbor a similar system. Interestingly, it has  
529 been hypothesized by Berglund *et al.* (Berglund *et al.* 2010b) that this GTA element  
530 allows large-scale gene transfer not only between *Bartonella* strains infecting the  
531 same host or vector simultaneously, but also between strains present at different  
532 time points. In our study, clear footprints of lateral gene transfer highlighted those  
533 individual genotypes which had received foreign DNA relatively recently, and also

534 those genotypes likely to be the common ancestor of an entire lineage as they  
535 occurred early in the branch, as shown for *B. taylorii* clusters B and C. Our analysis  
536 also demonstrated DNA exchanges between *B. taylorii* clusters A and D; and *B.*  
537 *taylorii* cluster D and *B. grahamii* cluster G. Interestingly, recombination between  
538 wood mice-specific genotypes (cluster E) and bank vole-specific genotypes from *B.*  
539 *taylorii* and *B. grahamii* have also been highlighted, suggesting their co-occurrence in  
540 a host or vector during their life cycle. Interestingly, different rodent species in the  
541 Sénart forest had equivalent diversity of rodent flea communities, supporting the  
542 hypothesis of genetic exchange within the vector (Pisanu *et al.* 2008).

543         In order to evaluate the potentially increased risk for humans to be infected  
544 with rodent-adapted *Bartonella* species due to possible host-shifting, understanding  
545 the molecular mechanisms of host specificity in areas where humans encounter  
546 rodents is extremely important. As for the mechanisms responsible for host  
547 specificity, our results clearly indicate that the *virB5* phylogeny is not sufficient to  
548 completely account for the pattern of rodent host association, and therefore must  
549 depend on additional more complex systems. Recent genomic surveys have  
550 identified many other putative host adaptability genes essential for blood stream  
551 infection (Engel *et al.* 2011; Saenz *et al.* 2007) including other T4SS, adhesions,  
552 autotransporters, etc, suggesting that a global analysis of all genomes is required in  
553 order to elucidate the subtle differences between closely related genotypes with  
554 different host ranges. Our study provides a unique set of data that allows broad  
555 genome comparison between very closely related *Bartonella* strains or genotypes  
556 (such as clusters E and B), isolated in their natural environment, and with different  
557 host specificity patterns, to further elucidate *Bartonella* host range preference at the  
558 molecular level.

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563

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572

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708

709

710 **Tables**

711

712 **Table 1.** Repartition of *Bartonella* species within the Sénart forest rodent community  
713 according to their genotypes.

714

Host	n	<i>B. taylorii</i>					<i>B. grahamii</i>	<i>B. rochalimae</i> -like	<i>B. doshiae</i>
		A	B	C	D	E	G	H	F
Field voles	9	0	0	0	1	0	0	0	0
Bank voles	471	49	28	31	42	0	28	1	6
Wood mice	70	3	0	0	0	6	0	0	0

716

717

718 **Table 2.** Polymorphism and nucleotide diversity of six housekeeping protein-coding  
719 genes among *Bartonella* strains720  $\pi$ , average number of nucleotide differences per site; dS, number of synonymous  
721 changes per synonymous site; dN, number of non-synonymous changes per non-  
722 synonymous site; NA, not applicable

Genes	Size (bp)	N. alleles	Polymorphic (non synonymous) sites		dN	dS	dN/dS	$\pi$ (%)
			N	%				
<b>All genotypes (n = 64)</b>								
<i>ftsZ</i>	788	8	147	18.65	0.0064	0.1623	0.0396	4.405
<i>gltA</i>	326	11	73	22.39	0.0128	0.2064	0.0619	5.419
<i>groEL</i>	812	28	217	26.72	0.0118	0.1520	0.0775	4.625
<i>nuoG</i>	325	16	56	17.23	0.0037	0.2262	0.0164	5.801
<i>ribC</i>	408	18	138	33.82	0.0428	0.1816	0.2358	7.562
<i>rpoB</i>	788	9	162	20.56	0.0080	0.1785	0.0449	4.952
Concatenate	3448	41	793	23.00	0.0127	0.1758	0.0724	5.182
<b><i>B. taylorii</i> (n = 48)</b>								
<i>ftsZ</i>	788	4	26	3.30	0.0001	0.0376	0.0037	0.920
<i>gltA</i>	326	7	32	9.82	0.0065	0.0790	0.0820	2.202
<i>groEL</i>	812	22	112	13.79	0.0101	0.0175	0.5784	1.199
<i>nuoG</i>	325	14	51	15.69	0.0047	0.1583	0.0296	4.208
<i>ribC</i>	408	13	98	24.02	0.0158	0.0597	0.2652	2.618
<i>rpoB</i>	788	6	90	11.42	0.0020	0.0570	0.0351	1.542
Concatenate	3448	33	409	11.86	0.0058	0.0548	0.1062	1.760
<b><i>B. grahamii</i> (n = 16)</b>								
<i>ftsZ</i>	788	2	1	0.13	0.0002	0.0000	-	0.016
<i>gltA</i>	326	2	2	0.61	0.0005	0.0018	0.2722	0.077
<i>groEL</i>	812	4	9	1.11	0.0002	0.0114	0.0175	0.296
<i>nuoG</i>	325	1	0	0.00	0.0000	0.0000	-	0.000
<i>ribC</i>	408	3	17	4.17	0.0050	0.0070	0.7133	0.547
<i>rpoB</i>	788	2	1	0.13	0.0000	0.0024	0.0000	0.058
Concatenate	3448	7	30	0.87	0.0007	0.0043	0.1733	0.159

723

724 **Figure legends**

725 **Fig. 1.**

726 (a) Maximum-likelihood analysis of 63 *Bartonella* unique genotypes and 19  
727 *Bartonella* species detected in this study, using the alignment of concatenated  
728 sequences of six loci (*ftsZ*, *gltA*, *groEL*, *nuoG*, *ribC* and *rpoB*). The numbers at the  
729 nodes correspond to bootstrap values higher than 80%.

730 (b) Results of Structure analysis showing the inferred proportion of nucleotides from  
731 four ancestral populations for each strain. The plot shows one vertical line for each  
732 strain, and the length of the colored segments indicates the proportions of  
733 nucleotides from each of the four ancestral populations.

734

735 **Fig. 2.**

736 Phylogeny of *virB5* gene sequences using Maximum likelihood, with a GTR  
737 substitution model. Clusters in which *virB5* alleles were found are indicated by colors  
738 and shapes identical to Fig. 1 (see legend). Bootstrap values higher than 80% are  
739 given at the nodes. Arrows indicate *virB5* sequences amplified and sequenced from  
740 strains obtained from wood mice.

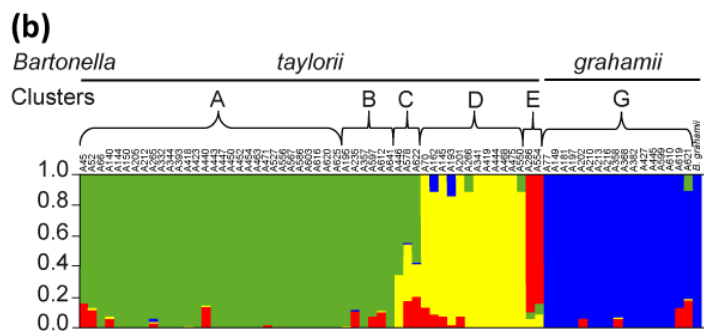
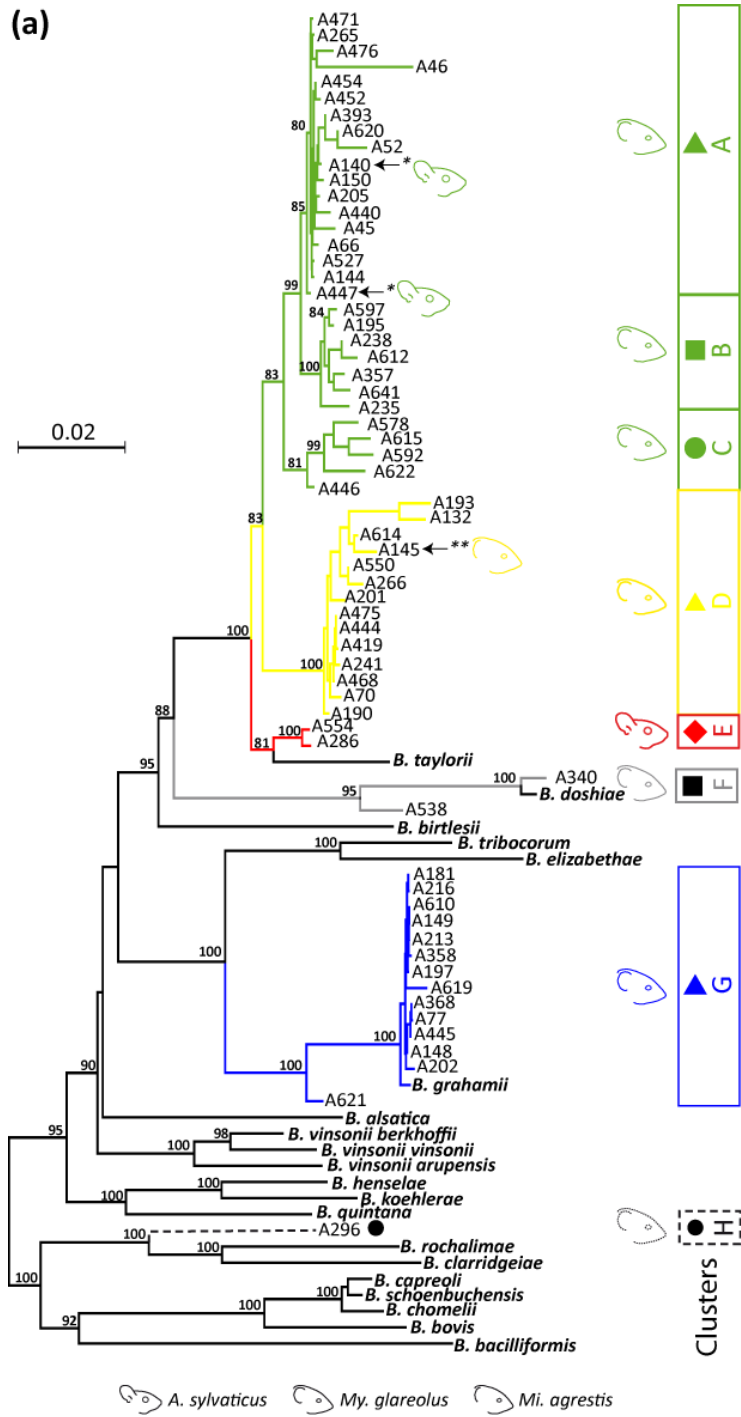


Fig. 1



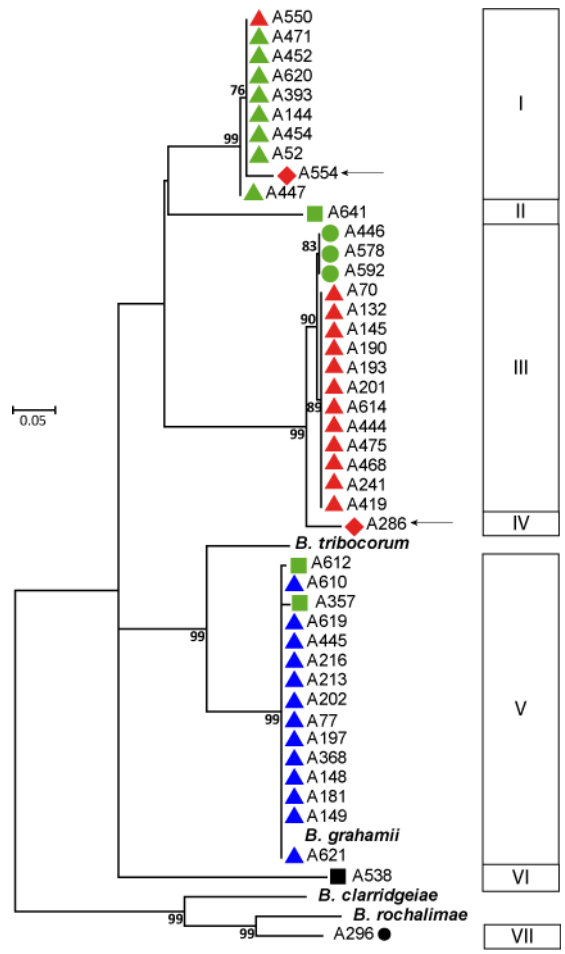


Fig. 2

745

746 **Supplementary Table S1**747 GenBank accession numbers for *ftsZ*, *gltA*, *groEL*, *ribC*, *rpoB* and *nuoG* sequences

Species	Strain	Accession numbers						748
		<i>ftsZ</i>	<i>gltA</i>	<i>groEL</i>	<i>ribC</i>	<i>rpoB</i>	<i>nuoG</i>	
<i>B. taylorii</i>	M6	AF467756	Z70013	AF304017	AY116635	AF165995	EF659943	
<i>B. doshiae</i>	R18	AF467754	AF207827	AF014832	AY116627	AF165991	-	
<i>B. birtlesii</i>	IBS 325	AM690313	AF204272	AM690315	AM690314	AB196425	-	
<i>B. tribocorum</i>	CIP 105476	NC_010161	NC_010161	NC_010161	NC_010161	NC_010161	NC_010161	
<i>B. elizabethae</i>	F9251	AF467760	Z70009	AF014834	AY116633	AF165992	EF659940	
<i>B. grahamii</i>	as4aup	NC_012846	NC_012846	NC_012846	NC_012846	NC_012846	NC_012846	
<i>B. alsatica</i>	IBS 382	AF467763	AF204273	AF299357	AY116630	AF165987	EF659935	
<i>B. vinsonii</i> subsp. <i>vinsonii</i>	Baker	AF467757	Z70015	AF014835	AY116636	AF165997	EF659944	
<i>B. vinsonii</i> subsp. <i>arupensis</i>	OK94-513	AF467758	AF214557	AF304016	AY116631	AY166582	EF659936	
<i>B. vinsonii</i> subsp. <i>berkhoffii</i>	93-CO1	AF467764	BVU28075	AF014836	AF548031	AF165989	EF659937	
<i>B. henselae</i>	Houston-1	NC_005956	NC_005956	NC_005956	NC_005956	NC_005956	NC_005956	
<i>B. koehlerae</i>	C-29	AF467755	AF176091	AY116641	AY116634	AY166580	EF659942	
<i>B. quintana</i>	Toulouse	NC_005955	NC_005955	NC_005955	NC_005955	NC_005955	NC_005955	
<i>B. rochalimae</i>	ATCC BAA-1498	FN645461	FN645459	FN645466	-	FN645459	FN645459	
<i>B. clarridgeiae</i>	73	NC_014932	NC_014932	NC_014932	NC_014932	NC_014932	NC_014932	
<i>B. capreoli</i>	IBS 193	AB290192	AF293392	AB290190	AB290194	AB290188	-	
<i>B. schoenbuchensis</i>	R1	AF467765	AJ278183	AY116642	AY116628	AY167409	FN645509	
<i>B. chomelii</i>	A828	AB290193	AY254308	AB290191	AB290195	AB290189	-	
<i>B. bovis</i>	91-4	-	AF293394	-	AY116637	AY166581	EF659938	
<i>B. bacilliformis</i>	KC583	NC_008783	NC_008783	NC_008783	NC_008783	NC_008783	NC_008783	

28

749

750 **Supplementary Fig. S1.**

751 Individual phylogenies of *Bartonella* genotypes constructed using internal sequences  
752 of six protein-coding genes (*ftsZ*, *gltA*, *groEL*, *nuoG*, *ribC* and *rpoB*). Colors and  
753 shapes are identical to Fig. 1 (see legend). Genotypes with positions that are  
754 discordant with the concatenate-based phylogeny are indicated on each phylogeny;  
755 and are colored according to their position in the concatenated phylogeny. Bootstrap  
756 values higher than 75% are given at the nodes.

757

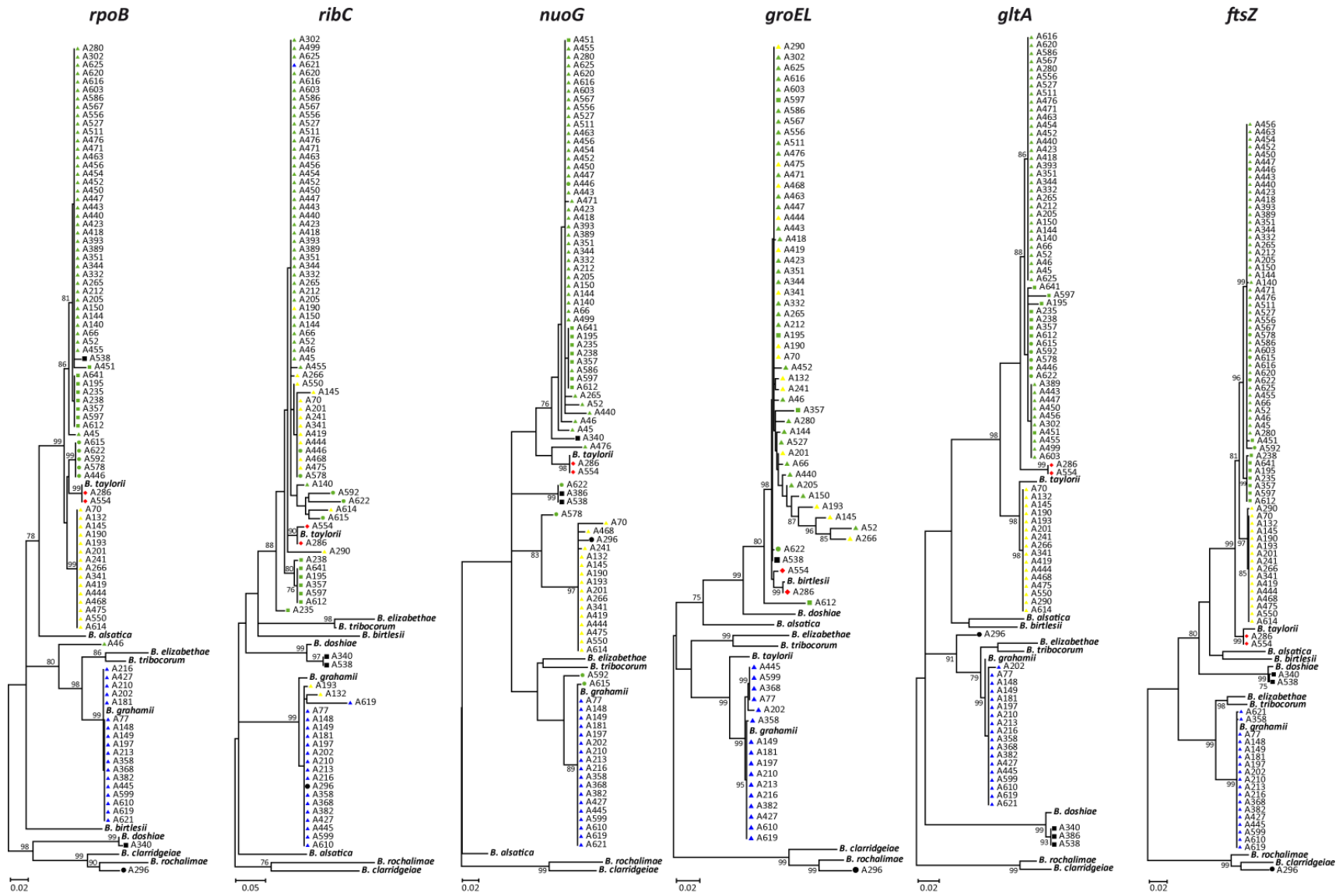
758 **Supplementary Fig. S2.**

759 50% consensus tree from ClonalFrame.

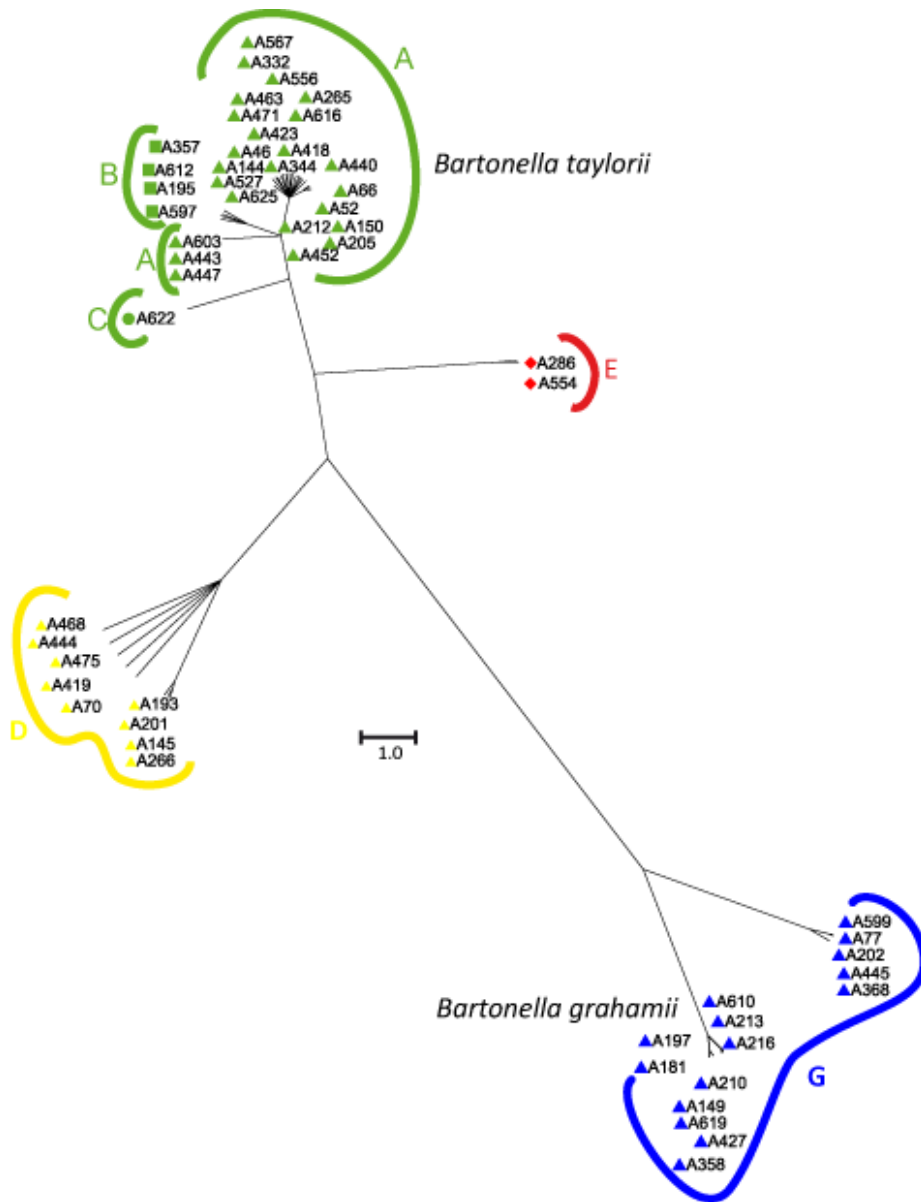
760

761 **Supplementary Fig. S3.**

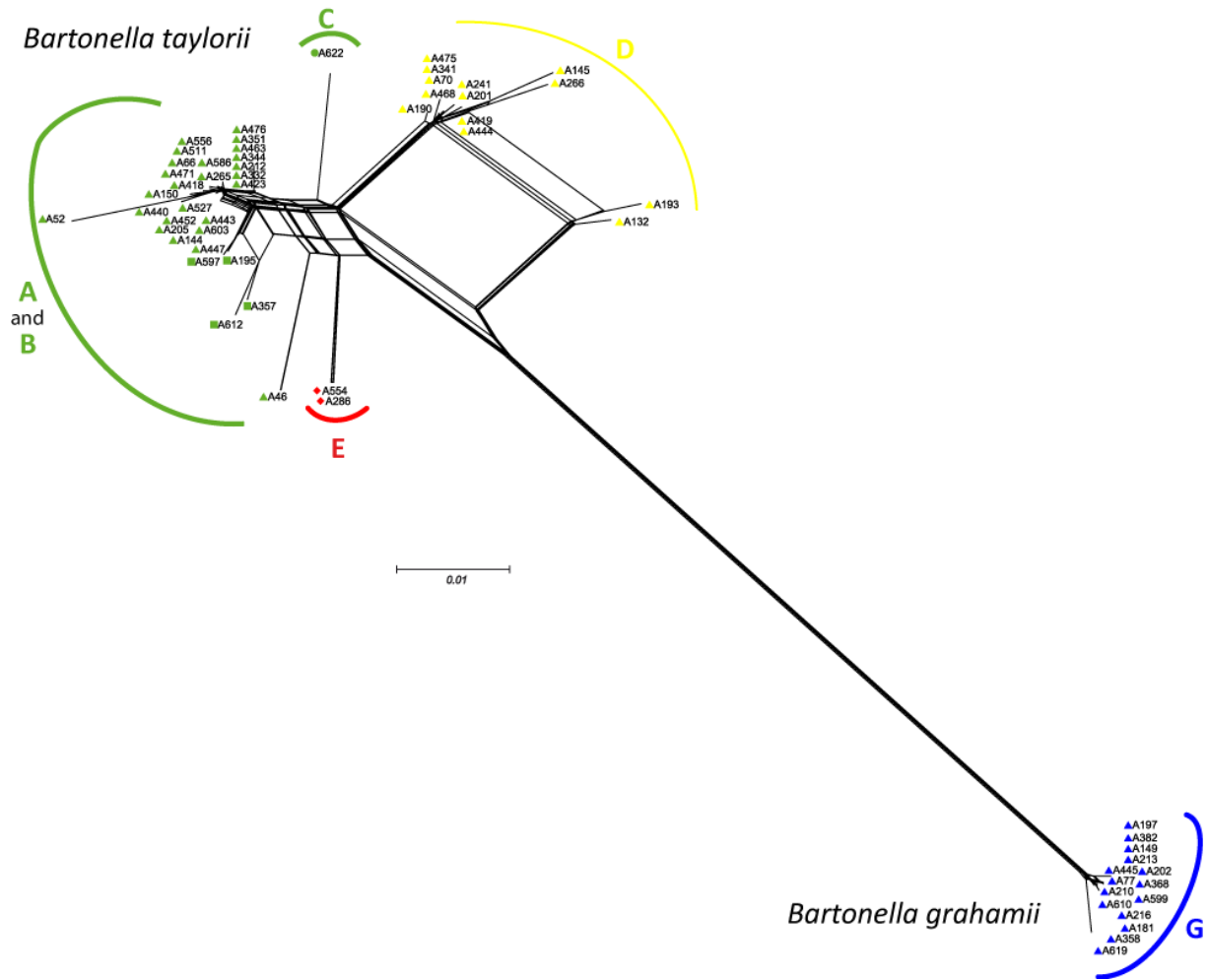
762 Neighbor-Net graph based on concatenated sequences of the six housekeeping  
763 genes of *Bartonella* strains. Note the bushy network structure among *Bartonella*  
764 strains indicative of pervasive homologous recombination.



Supplementary fig. S1



Supplementary Fig. S2



Supplementary Fig. S3

## Conclusions

Parmi la population totale de rongeurs, 193 (35,4%) individus se sont révélés porteurs de bartonelles. L'analyse phylogénétique de ces 193 souches a mis en évidence 63 génotypes distincts affiliés à 3 espèces, *B. taylorii*, *B. grahamii* et *B. doshiae*, fréquemment retrouvées au sein des campagnols et des mulots sur tout le continent eurasiatique [25, 28-30], et à une nouvelle espèce proche de *B. rochalimae*. La majorité des génotypes obtenus au cours de cette étude appartiennent aux espèces *B. taylorii* et *B. grahamii* comptabilisant 158 et 28 souches respectivement. Au-delà de l'espèce, les génotypes affiliés à *B. taylorii* sont structurés en 5 groupes ou clades bien distincts et présentent une diversité nucléotidique élevée au sein des gènes de ménage, alors que les génotypes de *B. grahamii* sont relativement homogènes, formant un unique groupe sans structure phylogénétique marquée. Au sein des souches de *B. grahamii*, la restriction de diversité et la taille de la population sont caractéristiques d'un goulot d'étranglement, profitant vraisemblablement aux souches de *B. taylorii* qui, à l'inverse, se diversifient en différentes lignées pour occuper les niches écologiques (hôtes ou vecteurs) laissées vacantes. L'analyse des séquences *virB5* confirme ce phénomène en indiquant une grande diversité de séquences pour les souches de *B. taylorii* et une homogénéité au sein des souches de *B. grahamii*. Étant donné que la plupart des génotypes étaient hébergés par les campagnols roussâtres, nous avons émis l'hypothèse que le goulot d'étranglement subi par les souches de *B. grahamii* serait dû à une transmission d'hôte à hôte restreinte à un seul type de vecteur, alors que les souches de *B. taylorii* seraient capables d'être vectorisées par une plus grande diversité de puces.

En comparant le portage des génotypes de *Bartonella* par les différentes espèces de rongeurs collectées, nous avons démontrés que le cortège de *Bartonella* est bien plus diversifié au sein des campagnols roussâtres qu'au sein des campagnols agrestes ou des mulots. Nous avons aussi constaté que seuls les campagnols roussâtres hébergeaient l'intégralité des génotypes identifiés durant cette étude, à l'exception de 2 génotypes, qui se regroupent en 1 clade distinct au sein de l'espèce *B. taylorii* et qui ont été retrouvés exclusivement chez certains mulots. De plus, quelques génotypes au sein de 2 clades de *B. taylorii* sont hébergés d'une part, chez le campagnol roussâtre et chez le campagnol agreste et d'autre part, chez le campagnol roussâtre et le mulot. Ces résultats indiquent donc une



spécificité d'hôte pour la majorité des génotypes obtenus lors de cette étude. En revanche, la phylogénie des séquences *virB5* ne reflète pas cette spécificité d'hôte confirmant que l'interaction des espèces de *Bartonella* à leur hôte rongeur ne dépend pas uniquement de la protéine VirB5. En effet, l'association des espèces de *Bartonella* avec leur hôte est régit par un système bien plus complexe, faisant intervenir de nombreux autres gènes codant pour des protéines impliquées dans l'adhésion et l'invasion des cellules endothéliales et des globules rouges de l'hôte (d'autres composants des SST4, des adhésines, des autotransporteurs, ...) [31-32].

En utilisant les six gènes de ménages, nous avons démontré que les transferts horizontaux ont bien plus contribué à générer de la diversification au sein des souches de *Bartonella* que les mutations spontanées. De plus, il s'avère que les événements de recombinaison homologue sont bien plus fréquents au sein de *B. taylorii* que de *B. grahamii*, ce qui corrèle avec la différence de diversité observée entre les 2 espèces. Par la suite, la visualisation des transferts horizontaux nous a permis de constater, d'une part, que les génotypes de *B. taylorii* et *B. grahamii* strictement associées aux campagnols roussâtres recombinent entre eux. D'autre part, les génotypes de *B. taylorii* exclusivement adaptées aux mulots recombinent avec les génotypes de *B. taylorii* et *B. grahamii* associées aux campagnols roussâtres. Malgré la spécificité d'hôte observée auparavant, ces transferts horizontaux entre les différents génotypes sont la preuve de leur co-présence à un moment donné au cours de leur cycle de vie.

Cette étude est la première du genre en Europe à décrire la diversité des souches de *Bartonella* aussi finement pour établir clairement la spécificité d'hôte vis-à-vis des espèces de rongeurs sylvatiques. De plus, lors de ce travail, nous avons obtenu une collection unique de souches à partir d'une population naturelle de rongeurs que nous pourrions utiliser pour des analyses complémentaires afin d'explorer les mécanismes moléculaires impliqués dans cette spécificité d'hôte.

**Chapitre III.**  
**Co-infection par *Borrelia afzelii* et**  
***Bartonella* spp. au sein d'une**  
**population de campagnols roussâtres**

## Chapitre III.

# Co-infection par *Borrelia afzelii* et *Bartonella* spp. au sein d'une population de campagnols roussâtres (Article 2)

### Introduction

Dans le second chapitre de cette thèse, nous avons démontré une homogénéité parmi les souches de *B. grahamii*, et à l'inverse, une diversification des souches de *B. taylorii*. Nous avons aussi montré que toutes les souches de *Bartonella* peuvent recombinaison et ont donc l'opportunité d'interagir entre elles, malgré une barrière physique liée à la spécificité d'hôte. Nous en avons alors déduit que le deuxième hôte des bartonellae, le vecteur arthropode, joue un rôle majeur dans la structuration des souches. Cependant, d'autres facteurs peuvent avoir un impact sur la diversité et la dynamique des souches de *Bartonella*. En effet, en Angleterre, il a été prouvé par des tests statistiques robustes et par un échantillonnage conséquent que l'infection des campagnols agrestes par *B. taylorii* module, et est modulée par l'infection d'autres bactéries, virus et protozoaires [33]. Les auteurs ont montré que la présence du cowpox virus favorisait la susceptibilité des campagnols vis-à-vis de *B. taylorii*, et augmentait la durée de la bactériémie. Au contraire, l'infection de ces campagnols par *Babesia microtii* et *Anaplasma phagocytophylum* impactait de façon négative sur l'infection par *B. taylorii*, certainement à cause d'une réponse immunitaire croisée et/ou d'une compétition pour des ressources communes [33]. De nombreux autres microorganismes peuvent infecter les espèces de micromammifères, et peuvent donc potentiellement interagir avec les espèces de *Bartonella*.

La présence de l'espèce bactérienne *Borrelia afzelii* au sein des campagnols roussâtres a déjà été décrite dans la zone d'étude (la forêt de Sénart) [34]. En Europe, la bactérie *Borrelia afzelii*, appartenant au complexe d'espèce *Borrelia burgdorferi* sensu lato et agent de la borréliose de Lyme, infecte fréquemment les rongeurs sylvatiques, incluant le mulot sylvestre et le campagnol roussâtre [34-35].

Lors de cette étude, nous avons donc cherché à savoir si l'infection par *Borrelia afzelii* au sein d'une population de campagnols roussâtres a un effet sur la présence et la diversité des espèces de *Bartonella*.



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## Co-infection of *Borrelia afzelii* and *Bartonella* spp. in bank voles from a suburban forest

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### ABSTRACT

We report the molecular detection of *Borrelia afzelii* (11%) and *Bartonella* spp. (56%) in 447 bank voles trapped in a suburban forest in France. Adult voles were infected by significantly more *Borrelia afzelii* than juveniles ( $p < 0.001$ ), whereas no significant difference was detected in the prevalence of *Bartonella* spp. between young and adult individuals ( $p = 0.914$ ). Six percent of the animals were co-infected by both bacteria. Analysis of the bank vole carrier status for either pathogen indicated that co-infections occur randomly ( $p = 0.94$ ,  $CI_{95} = [0.53; 1.47]$ ). Sequence analysis revealed that bank voles were infected by a single genotype of *Borrelia afzelii* and by 32 different *Bartonella* spp. genotypes, related to three known species specific to rodents (*B. taylorii*, *B. grahamii* and *B. doshiae*) and also two as yet unidentified *Bartonella* species. Our findings confirm that rodents harbor high levels of potential human pathogens; therefore, widespread surveillance should be undertaken in areas where humans may encounter rodents.

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Rodents are recognized as a major reservoir of pathogens such as tick-borne diseases, which can cause serious illness in livestock and humans [1]. Tick-borne diseases are extraordinarily diverse and are responsible for high morbidity and mortality rates around the world. *Ixodes ricinus*, the most widespread and abundant tick in Western Europe, accounts for significant numbers of human bites and is a major vector of pathogens responsible for rodent-borne disease [2,3].

In Europe, the most prevalent tick-borne disease is Lyme borreliosis, caused by bacteria belonging to the *Borrelia burgdorferi* sensu lato (s.l.) complex [4]. Particular species of rodents are known to be infected by certain *Borrelia* species. *Borrelia afzelii* has been described as specific to smaller rodent species, including the wood mouse (*Apodemus sylvaticus*) and bank vole (*Myodes glareolus*), while *B. burgdorferi* sensu stricto is found in other rodent hosts, such as the red squirrel (*Sciurus vulgaris*) and brown rat (*Rattus norvegicus*) [5–7]. These *Borrelia* species are known causative agents of human disease [8] but in addition, they can assume an aggravating role when co-infected along with *Babesia* sp., *Anaplasma* sp., *Ehrlichia* sp., and *Bartonella*

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sp. in Lyme disease [9,10]. However the prevalence of these co-infections remains unknown as their diagnosis is exceedingly difficult.

At least ten of the 26 *Bartonella* spp. are asymptotically carried by rodents, and five of the rodent-specific *Bartonella* spp. (*B. grahamii*, *B. elizabethae*, *B. vinsonii* subsp. *arupensis*, *B. washoensis* and *B. tamiae*) have been identified as zoonotic agents [11]. These bacteria induce diverse and species-dependent diseases in humans, but mostly cause bacteremia, myocarditis, endocarditis, lymphadenitis and hepatitis [12]. *Bartonella* spp. are mostly transmitted to rodents by vectors such as fleas (*Ctenophthalmus nobilis nobilis* for voles) [13] and to a lesser extent, ticks (*I. ricinus*) [14].

In recent times, outdoor activities in suburban forests have been increasing, consequently providing excellent conditions for contact between rodents, arthropod vectors and humans. Thus for the exposed human population, there is an urgent need to conduct studies investigating the numbers of rodents carrying vector-borne zoonotic pathogens. Therefore this study was undertaken to obtain an initial assessment of the prevalence, diversity and co-infection rates of *B. burgdorferi* sensu lato and *Bartonella* spp. within a population of bank voles (*M. glareolus*), the most abundant diurnal murid species in the studied site, a highly visited suburban forest [15].

## 1. Material and methods

### 1.1. Study site, trapping and animal processing

The study was conducted in the Sénart Forest (3200 ha, 02°29'E, 48°40'N), which is located 22 km from Paris (France) between the months of March and October over a four-year period (2006–2009). This forest is essentially composed of broad-leaved trees dominated by oaks (*Quercus robur*, *Quercus pedunculata*) and European hornbeams (*Carpinus betulus*), with two other forest tree species, the sweet chestnut (*Castanea sativa*) and the lime tree (*Tilia vulgaris*). Infection prevalence was studied in 447 bank voles trapped within a grid system consisting of 104 geo-localized Sherman<sup>®</sup> baited traps. For all captured individuals, sex was determined and the age class (juvenile and adult) evaluated by coupling the date of capture and the body weight. For *B. burgdorferi* s.l. detection, ear biopsy was obtained by cutting a small piece from the ear of the trapped voles (maximum 3 mm<sup>2</sup>) with scissors, then immediately storing the sample in 90% ethanol [7]. The bank voles were then sacrificed and their spleens removed under sterile conditions and stored at –80 °C until they were to be used for *Bartonella* spp. detection.

### 1.2. DNA extraction

For *Borrelia* analysis, DNA from one ear biopsy per bank vole was extracted using the NucleoSpin<sup>®</sup> Tissue kit (Machery-Nagel, Düren, Germany) [7].

The presence of *Bartonella* spp. was detected by extracting DNA from spleen samples of each bank vole. First, a piece of the sample was ground in 500 µl of F-12 nutrient

mixture medium (Invitrogen) and 250 µl of this mixture were placed in a Lysing Matrix E tube (MP Biomedical, Solon, USA). Then, 500 µl of hexadecyltrimethylammonium bromide (CTAB) extraction buffer and 500 µl of phenol–chloroform–isoamyl alcohol (25:24:1; pH 8.0) were added to the sample. Extraction buffer was prepared by mixing equal volumes of 10% (w/v) CTAB (Sigma–Aldrich, France) in 0.7 M NaCl with 240 mM potassium phosphate buffer, at pH 8.0. Samples were homogenized and lysed for 30 s at a speed setting of 6500 rpm in a Precellys 24 centrifuge (Bertin Technologies, Montigny-le-Bretonneux, France). The aqueous phase, containing nucleic acids, was separated by centrifugation at 16,000 × g for 5 min at 4 °C, and transferred to a new microcentrifuge 2 ml tube. Phenol was extracted by adding an equal volume of chloroform–isoamyl alcohol (24:1), mixing the tubes by inversion then centrifuging for 5 min at 16,000 × g (4 °C). The nucleic acids present in the aqueous phase were precipitated by adding two volumes of 30% (w/v) polyethylene glycol (Fluka Biochemica, Sigma–Aldrich, France) – 1.6 M NaCl solution for 2 h at room temperature. The tubes were then centrifuged for 10 min at 18,000 × g (4 °C). The pelleted nucleic acids were washed in ice-cold 70% (v/v) ethanol and then centrifuged at 10,000 × g for 3 min (4 °C). The ethanol was eliminated, and the pellets were air dried, and then resuspended in 50–200 µl of Tris–EDTA buffer (pH 7.4), depending on their size, and stored overnight at 4 °C.

### 1.3. PCR amplification and sequencing

PCR analysis for *B. afzelii* was carried out using DNA extracted from the ears, and *Bartonella* spp. detection was performed using DNA extracted from the spleen.

Each reaction was carried out in a total volume of 25 µl containing: 0.5 mmol/ml of each primer, 2.5 mM of each dNTP, 2.5 µl of 10× PCR buffer, 1 U of Taq DNA polymerase (Takara Biomedical Group, Shiga, Japan) and 5 µl of the DNA extract. The PCR was performed in a MyCycler thermocycler (Bio-Rad, Strasbourg, France). Negative and positive controls were included in each run, (water and then either genomic DNA of *B. burgdorferi* sensu stricto isolated in our laboratory or genomic DNA of *B. birtlesii* strain IBS 325).

The presence of *B. afzelii* in the extracted DNA has been detected using a PCR that targets the *rrs* gene (16S rRNA) [7]. The intergenic 16S(*rrs*)–23S(*rrlA*) spacer (IGS) was then used in this study to characterize the diversity of *B. afzelii* as previously described [16].

The presence of *Bartonella* spp. was determined by using a portion of the *gltA* gene encoding for the citrate synthase protein, previously shown to be a reliable tool for distinguishing *Bartonella* spp. and closely related genotypes [17]. The *gltA* fragment was amplified by PCR using primers specific to the *Bartonella* genus [18].

The amplification products were analyzed by electrophoresis on 1% agarose gels with 0.1 mg/ml of ethidium bromide and imaged under UV light. PCR products obtained from positive samples were then purified and sequenced in both directions by Eurofins MWG Operon (Germany).



#### 1.4. Phylogenetic analysis and construction of phylogenetic trees for *gltA* and IGS sequences

Sequences were analyzed using the sequence analysis software Lasergene (DNASTAR, Madison, WI, USA), which determined the consensus sequence for the amplified region of the *gltA* gene and the intergenic 16S(*rrs*)–23S(*rrlA*) spacer. Sequence data were aligned with those of typed strains of *Bartonella* and *Borrelia* species using CLUSTAL W software (included in MEGA 5). MEGA 5 software (<http://www.megasoftware.net/>) [19] was used to calculate the DNA sequence similarities (P-distance). Phylogenetic trees were constructed using the neighbor joining (NJ) method, combined with the Jukes-Cantor parameters method. Bootstrap analysis was carried out on 1000 replications. Sequences were also analyzed by the BLASTn algorithm [20] to identify the closest relative.

#### 1.5. Data analysis

Co-infections by *B. afzelii* and *Bartonella* spp., as well as rodent age compared to the carrier-status for each pathogen, were statistically evaluated using the chi-squared test and the software Epi Info version 3.5.3 (CDC, Atlanta, GA, USA). A two-tailed Fisher's exact test was used when the expected numbers of observations were less than five. A *p*-value of <0.05 was considered to be statistically significant. The diversity of *Bartonella* spp. sequences was calculated using the Hunter and Gaston discrimination index [21].

## 2. Results

*B. afzelii rrs* was detected in 49 of the 447 bank voles (11%), and infected more adults than juveniles, at levels of 14.4% and 1.7% respectively ( $p < 0.001$ ). IGS sequences were all identical (100% identity – accession number: JQ693994) and shared 93.8% identity with the *B. afzelii* IGS sequence amplified from *I. ricinus* collected in southern Sweden (accession number AY363694) [16]. The phylogenetic tree based on the IGS fragment showed that the unique *B. afzelii* sequence identified here is distant from all other IGS *B. afzelii* sequences amplified from rodents, ticks and humans from Sweden, UK and Latvia, with a pairwise sequence dissimilarity that ranged between 4.8% and 6.4% (Fig. 1).

The *Bartonella* spp. specific fragment of the *gltA* gene was amplified from 252/447 bank voles (56.4%). Prevalence rates were similar in juveniles (56.7%) as in adults (56.3%) ( $p = 0.915$ ,  $CI_{95} = [0.72; 1.34]$ ). Sequence analysis of *gltA* fragments revealed the presence of 32 different *gltA* genotypes (unique sequence variants with  $\geq 1$  nucleotide difference). The percentage identity of the *gltA* sequence with known *Bartonella* type strains ranged from 91 to 100%. Simpson's index was 0.85, indicating that a diverse range of *Bartonella* spp. *gltA* amplicons was found in the bank voles. A phylogenetic tree based on the partial sequence of the *gltA* gene is shown in Fig. 2. The 32 *gltA* genotypes were distributed in four clades along the *gltA* dendrogram, i.e., A, B, C and D (Fig. 2), and which are detailed as follows.

Of these 32 genotypes, 12 belonged to clade A (A32RCG, A42FCG, A60RCG, A252FCG, A286FCG, A343RCG, A351FCG,

A364FCG, A479FCG, A573FCG, A597FCG, A641RCG). They showed 95.9 to 98.8% similarity to *Bartonella taylorii* type strain (AF191502). Those genotypes were closely related (97–100% similarity) to *Bartonella* strains (AY435106, AY435113, AF391790 and GU338962) isolated from field mice (*Apodemus flavicollis*) live-trapped in Greece, Sweden and Poland [22–24].

Clade B contained 11 genotypes, 10 of which (A8RCG, A53RCG, A55RCG, A138RCG, AR183RCG, A186RCG, A191RCG, A306RCG, A321FCG, A373FCG) were related to a *Bartonella* strain (FJ946856) recovered from stray dogs in Thailand [25] with 96.2–100% similarity. One genotype (A188RCG) represented a unique sequence which differed from all previously described *Bartonella* strains or sequences (<95% identity).

Clade C contained seven genotypes. Six of which (A142R, A143R, A216F, A296F, A509F and A582R), exhibited the closest phylogenetic relationship (94–100%) to *B. grahamii* (Z70016), a bacterium isolated from various rodent species worldwide [26] and potentially pathogenic to humans [27]. One genotype from clade C (A199RCG) also represented a unique sequence which differed from all previously described *Bartonella* strains or sequences (<92% identity).

Clade D clustered two genotypes (A543R and A636F), both closely related to a *B. doshiae* type strain (Z70017) (97.9 and 98.8% respectively), originally isolated from rodents in the United Kingdom [28].

The most frequently found *Bartonella* were genotypes belonging to clade A (closely related to *B. taylorii* type strain) detected in 58.8% (148/252) of the animals. Less frequent were genotypes from clade B in 21.4% (54/252) of the animals, genotypes A142R, A143R, A216F, A296F, A509F and A582R (from clade C and related to *B. grahamii*) in 16.3% (41/252) of animals, and genotypes of clade D (related to *B. doshiae*) in 2.8% (7/252) of animals. The two novel *Bartonella* genotypes A188R and A199R (from clades B and C) were each detected in only one specimen.

Co-infections were detected in 27/447 bank voles (6%) screened for the presence of *B. afzelii* and *Bartonella* spp. No trends of association or exclusion between the two bacteria were observed ( $\chi^2 = 0.4$ ,  $p = 0.849$ ,  $CI_{95} = [0.53; 1.47]$ ).

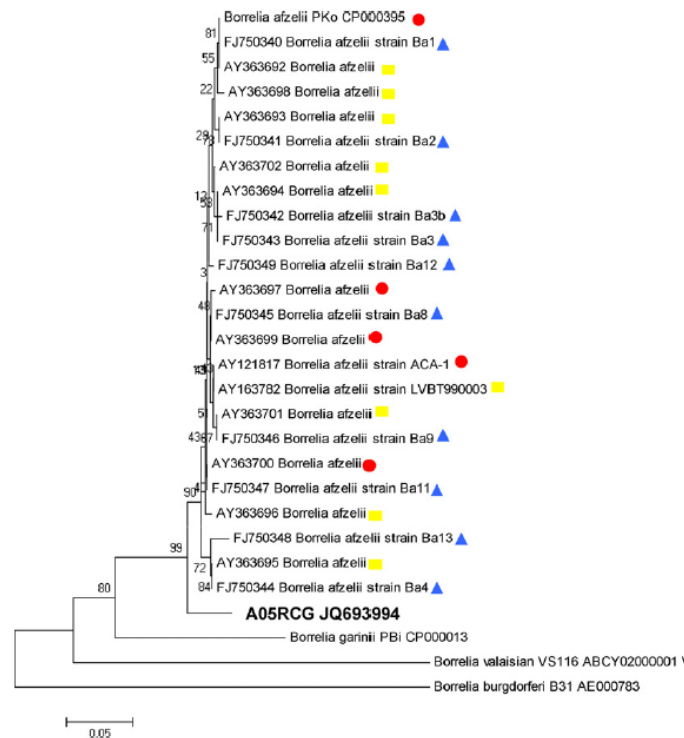
Co-infections occurred between *B. afzelii* and different *Bartonella* genotypes distributed throughout all clades. The *Bartonella* genotypes implicated in co-infections were, A351F and A364F (from clade A), closely related to *B. taylorii*; A373F, A8R and A53R, A188R belonging to clade B; A216F from clade C, which showed 100% relatedness to *B. grahamii* type strain, and A636F (clade D), with similarity to *B. doshiae*.

## 3. Discussion

This work is the first demonstration that bank voles are significantly infected with diverse *Bartonella* spp. (56.4%) and to a lesser extent with a single *B. afzelii* strain (11%), and also demonstrate a co-infection rate of 6%.

*B. afzelii* has already been shown to be present in rodents and some insectivore species at similar levels [4,29]. In our study, we identified one single genotype in bank voles in the geographical region studied. Interestingly, introduced Siberian chipmunks (*Tamias sibiricus*) and *I. ricinus* ticks

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**Fig. 1.** Phylogenetic relationships between the unique *Borrelia afzelii* genotype detected in the population of bank voles from the Sénart forest (France) and several *Borrelia* spp. type strains, based on the sequence of the intergenic 16S(*rrs*)-23S(*rrlA*) spacer (IGS). The phylogenetic tree was constructed by the neighbor-joining method. Bootstrap analysis was carried out on 1000 replications; values are indicated at the nodes. Genbank accession numbers are indicated for each sequence used. The sequence of *Leptospira biflexa* strain Patoc 1 (accession number: NC010502) was chosen as an outgroup in the phylogenetic tree. Symbols indicate the host origin of the amplified IGS sequence: (●) humans, (▲) rodents, (■) ticks. All sequences came from southern Sweden except PKo (CP000395) isolated in UK and LVB T990003 (AY163782) isolated in Latvia.

collected in the same site and during the same period as our study were found to harbor three different species in the *B. burgdorferi* complex: *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, suggesting that bank voles are exclusively tolerant to *B. afzelii* [7,30]. At various European sites, *B. afzelii* strains have been shown to exhibit restricted movement between geographic regions [31]. Moreover, Hellgreen and colleagues [32] showed that three different genetic loci of *B. afzelii* increased in stability as the geographical scale decreased, despite the occurrence of frequent recombination events. This suggests that specific *B. afzelii* strains are only associated with specific geographical regions, due to physical barriers and limited rodent movement. This could explain the low diversity of *B. afzelii* found in this study, as well as the differences between the *B. afzelii* genotypes reported here compared to those detected in other European countries [32].

Concerning *Bartonella* spp., the rodent infection rate observed in our study was similar to levels reported in many studies from different geographical areas: 52.4% in western USA [33], 62% in the United Kingdom [34], and 57% in Canada [35]. Conversely, studies conducted in southern

China and northern USA revealed *Bartonella* rodent infection rates exceeded 80% [36,37]. The presence of such high levels of *Bartonella* in rodent communities is still unexplained. In all these studies, as in ours, rodent-associated *Bartonella* species were genetically very diverse.

Our results also agree with those of Inoue and colleagues [26], who demonstrated that the different *B. grahamii* strains sampled from all over the world tend to be associated with a geographical region on a large scale, but that a strict association of strains with a specific rodent host did not seem to occur. In the bank vole population studied here, the genotypes from clade B, (except A188R) which formed a well-demarcated cluster (Fig. 2), were similar to a *Bartonella* sequences recovered from the blood of stray dogs in Thailand [25] and might represent a new species. Two unique genotypes, A188R from clade B and A199R from clade C showed less than 96% similarity with known *Bartonella* strains or sequences, suggesting the presence of two new species according to the criteria specified by La Scola et al. [17]. The exact taxonomic positions of these *Bartonella* genotypes now need to be further characterized by MLST or MLSA.

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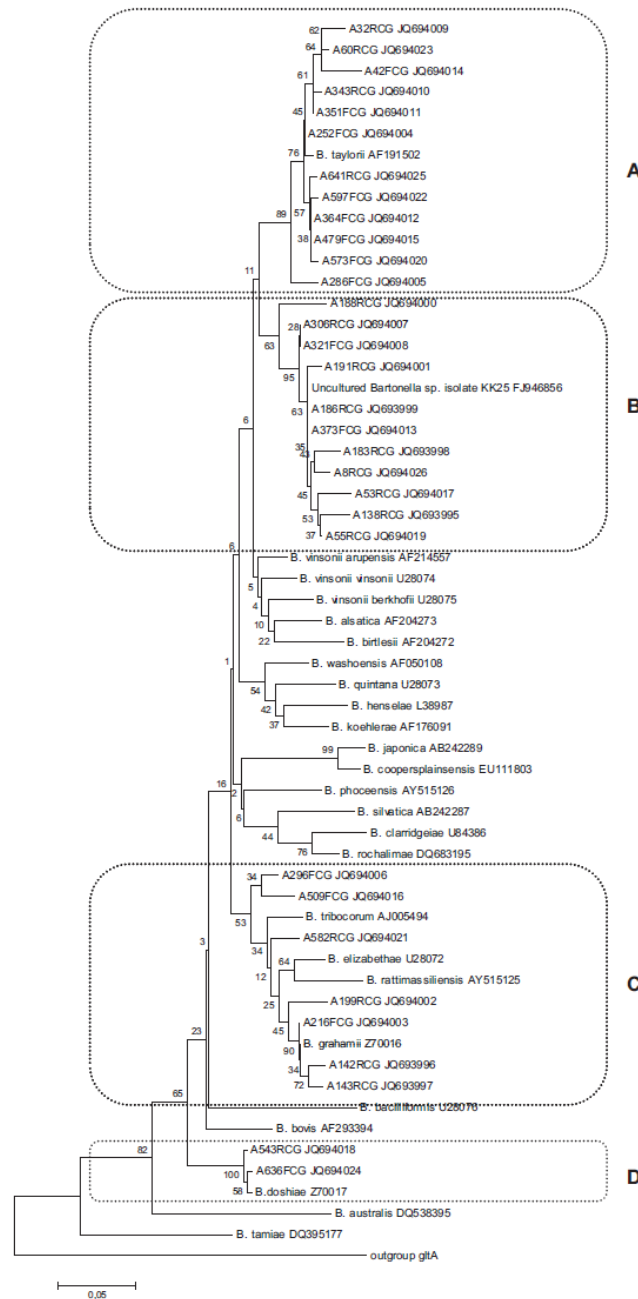


Fig. 2. Phylogenetic relationships among partial citrate synthase sequences (*gltA*) from the 32 *Bartonella* genotypes examined here and from known *Bartonella* species. The phylogenetic tree was constructed by the neighbor-joining method. Bootstrap values (percentage of 1000 replications) are indicated at the nodes. Genbank accession numbers are indicated for each sequence used. The sequence of *Brucella melitensis* strain 16M (accession number: NC003317) was chosen as an outgroup in the phylogenetic tree. The four clades that contained the genotypes recovered here are highlighted by boxes and labelled as follows: A, B, C, and D.

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We found a significant difference in the prevalence of *B. afzelii* between young and adult individuals. Juvenile bank voles are known to have lower levels of tick infestation compared to adults [38], most likely explained by the fact that juveniles inhabit a more confined physical environment, thereby limiting their exposure to *I. ricinus* [4]. In contrast to *B. afzelii*, we did not find any difference in *Bartonella* spp. prevalence in young and adult individuals. Hence, we hypothesize that the lack of difference between juveniles and adults suggests a similar exposure of both age groups to fleas, the major rodent *Bartonella* spp. vector, due to intimate contact between parents and their offspring in the burrow.

Wild populations are generally infected by more than one parasite type, which can result in different interactions between the parasites themselves, but also between the parasites and the host. Recent studies in a natural population of field voles (*Microtus agrestis*), demonstrated that the presence of cowpox virus increased vole susceptibility to *B. taylorii* infection, as well as the duration of bacteremia. In contrast, *Babesia microti* and *Anaplasma phagocytophilum* had a negative effect on *B. taylorii* infection probably due to an immune mediated response (cross immunity) and/or a competition for resources [39]. Six percent of the 447 bank voles screened here were co-infected with *Borrelia afzelii* and *Bartonella* genotypes. Considering that the *Bartonella* spp. bank vole infection rate with was around 56% in our study, half of the *Borrelia*-infected rodents were also infected with diverse *Bartonella* spp. suggesting that co-infections by the two bacteria occur randomly and are not correlated.

To our knowledge, this is the first study to assess the prevalence of both *Bartonella* and *Borrelia* species in rodents in a suburban forest, providing supplementary data about the geographic distribution and diversity of these vector-borne zoonotic bacteria. We also demonstrate the coexistence of both zoonotic pathogens in bank voles, but without apparent interactions. These preliminary data call for additional investigations to determine the impact of such pathogens on human health, especially when disease symptoms persist after exposure to rodents/arthropods for which no etiological agent has been identified.

#### Conflict of interests

The authors declared that they have no competing interests.

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## Conclusions

Les campagnols roussâtres sont infectés par diverses espèces de *Bartonella* (*B. taylorii*, *B. grahamii* et *B. doshiae*) avec une prévalence de 56,4%, et par un unique génotype de *Borrelia afzelii* présent dans 11% de la population. Nous avons aussi montré que les individus mûres étaient significativement plus infectés par *Borrelia afzelii* que les plus jeunes. Concernant les espèces de *Bartonella*, aucune différence significative de portage entre les jeunes et les adultes n'a été observée. Ces différences entre les deux bactéries proviennent probablement de leur mode de transmission. En effet, *Borrelia afzelii* est vectorisée par des tiques du genre *Ixodes*, alors que les espèces de *Bartonella* sont transmises au sein des rongeurs sylvatiques principalement par des puces. Les jeunes campagnols roussâtres sont moins infestés par les tiques que les adultes, car les juvéniles restent dans un endroit confiné, limitant leur exposition à *Ixodes ricinus* [36]. Au contraire, les juvéniles et les adultes seraient exposés de façon équivalente aux puces à cause du contact rapproché des parents et de leur progéniture dans le nid.

Parmi les 447 campagnols roussâtres analysés, 6% d'entre eux étaient co-infectés par *Borrelia afzelii* et par divers génotypes affiliés à *B. taylorii*, *B. grahamii* et *B. doshiae*, soit la moitié des individus infectés par *Borrelia afzelii*. En nous basant sur une inférence statistique, nous n'avons pas observé d'association ou d'exclusion entre les deux bactéries. Ces résultats nous indiquent donc que, au sein de la population de campagnols étudiée, les co-infections par *B. afzelii* et les espèces de *Bartonella* ne sont pas corrélées et semblent se produire au hasard sans interaction apparente entre les deux bactéries.



## Discussion générale et Perspectives

Cette thèse présentait comme objectif de contribuer à l'élucidation de l'écologie des souches de *Bartonella* adaptées aux micromammifères en Europe, en étudiant le niveau d'association qui lie ces souches à leur hôte et en déterminant l'influence de *Borrelia afzelii* sur la diversité des espèces de *Bartonella*. Dans un premier temps, l'étude de la diversité des souches de *Bartonella* circulant au sein d'une communauté de rongeurs sylvatiques dans une zone de forêt suburbaine française (la forêt de Sénart) nous a mené à explorer le degré de spécificité de ces souches vis-à-vis des espèces de rongeurs.

La spécificité d'hôte des espèces de *Bartonella* circulant au sein des communautés de rongeurs sylvatiques en Europe n'est pas clairement établie et reste encore aujourd'hui un sujet de controverse. La plupart des études visant à décrire la diversité des bartonellae au sein des espèces de rongeurs se sont basées sur le gène *gltA*. Or, il a été prouvé récemment que l'utilisation de ce gène est limitée pour résoudre les relations phylogénétiques entre les souches de *Bartonella* étroitement liées. De plus, au sein de *gltA*, des événements de recombinaison homologue peuvent avoir lieu entre des souches proches, voire entre différentes espèces, ayant pour conséquence une mauvaise identification de ces souches [37]. Ainsi le manque de précision dans la discrimination des souches pourrait avoir biaisé la déduction de la spécificité d'hôte dans les études ultérieures. L'utilisation de plusieurs gènes codant pour des protéines a été démontrée comme étant plus appropriée pour l'étude d'espèces bactériennes apparentées [38-39]. En effet, l'analyse des séquences de plusieurs gènes de ménage (Multilocus Sequence Analysis) procure un outil puissant pour délimiter des groupes de souches de *Bartonella* proches afin d'analyser leur distribution écologique [40-41].

Lors de l'étude constituant le second chapitre de cette thèse, nous avons donc utilisé les séquences de 6 gènes de ménages pour construire une phylogénie robuste et ainsi identifier précisément les 193 souches de *Bartonella* détectées et/ou isolées à partir d'une population de rongeurs constituées de campagnols roussâtres, de campagnols agrestes et de mulots sylvestres. Au sein de la population globale de rongeurs, cette phylogénie a permis l'identification d'un premier lot de 150 souches

structurées en 5 groupes ou clades appartenant à l'espèce *B. taylorii*, d'un second lot de 28 souches groupées en 1 seul clade correspondant à *B. grahamii*, de 6 souches affiliées à *B. doshiae* et d'une souche formant une nouvelle espèce à proximité de *B. rochalimae*.

Dans un premier temps, la taille restreinte de la population et l'homogénéité nucléotidique observées au sein des souches de *B. grahamii* sont caractéristiques d'un goulot d'étranglement. Le même phénomène a été observé au sein de souches de *B. grahamii* isolées à partir de campagnols et de mulots en Angleterre et en Suède [42]. Deux scénarios sont alors possibles. D'une part, comme il a été suggéré pour *B. quintana* [43], ce goulot d'étranglement serait le résultat d'un changement d'hôte relativement récent des souches de *B. grahamii* depuis la population de campagnols roussâtres vers un autre hôte rongeur sympatrique qui n'a pas été échantillonné lors de cette étude. Par exemple, le *Tamias* de Sibirie (*Tamias sibiricus*), introduit en France il y a une quarantaine d'année, colonise en partie la zone d'étude (la forêt de Sénart) partageant ainsi le même habitat que les campagnols roussâtres et les mulots sylvestres [44], et, en tant qu'espèce introduite, ce nouvel hôte serait donc susceptible d'être infecté par *B. grahamii*. D'autre part, le goulot d'étranglement subi par *B. grahamii* profiterait aux souches de *B. taylorii* qui semblent à l'inverse se diversifier en différentes lignées pour occuper les niches écologiques (hôtes ou vecteurs) vides. Etant donné que la majorité des génotypes de *B. taylorii* et de *B. grahamii* sont hébergés par les campagnols roussâtres, la transmission d'hôte à hôte pour les souches de *B. grahamii* serait restreinte à un seul type de vecteur alors que les souches de *B. taylorii* auraient la possibilité d'être transmises par une plus grande diversité de vecteurs. De plus, la communauté de puces infestant les campagnols roussâtres a été étudiée auparavant, et 3 espèces ont été identifiées, principalement *Ctenophthalmus agyrtes impavidus*, *Megabothris turbidus*, et dans une moindre mesure *Ctenophthalmus congener* (données recueillies par Benoît Pisanu et Jean-Louis Chapuis). L'étude de la diversité des espèces de *Bartonella* véhiculées par ces espèces de puces, ainsi que l'étude de leur compétence vis-à-vis des souches de *Bartonella* identifiées lors de ce travail, devrait nous permettre de mieux comprendre les différences de structure génétique observées entre *B. taylorii* et *B. grahamii*.

Par ailleurs, il serait aussi intéressant de caractériser les souches de *Bartonella* circulant au sein des autres espèces sympatriques de rongeurs en forêt

de Sénart afin de savoir si ces rongeurs sont capables d'acquérir les souches de *Bartonella* hébergées par les campagnols roussâtres et les mulots. Plus particulièrement, l'identification des souches de *Bartonella* circulant au sein des *Tamias* de Sibérie et d'écureuil roux (*Sciurus vulgaris*) permettrait de savoir si ces animaux hébergent des souches spécifiques de *B. washoensis*, distinctes des autres souches de *B. washoensis* isolées chez les espèces de sciuridés aux Etats-Unis d'Amérique et en Chine [41].

Dans un second temps, en mettant en relation les souches identifiées distinctement et les espèces de rongeurs à partir desquelles elles ont été isolées, nous avons constaté que la majorité des souches obtenue dans cette étude était hébergée exclusivement par le campagnol roussâtre, mise à part 2 génotypes regroupés au sein d'un même clade de *B. taylorii* et obtenus uniquement à partir de mulots. Ces résultats indiquent donc une forte spécificité des génotypes au sein de *B. taylorii*, *B. grahamii*, *B. doshiae* et *B. rochalimae*-like vis-à-vis des campagnols roussâtres et des mulots. Nous avons tout de même identifié quelques génotypes affiliés à *B. taylorii* qui présentaient un spectre d'hôte plus large, en ayant été retrouvés, d'une part, chez des campagnols roussâtres et un campagnol agreste, et d'autre part, chez des campagnols roussâtres et des mulots sylvestres.

Au niveau moléculaire, cette spécificité d'hôte est régit par plusieurs systèmes de sécrétion de type IV (SST4) qui permettent l'adhésion et l'invasion des cellules endothéliales de la niche primaire ainsi que des érythrocytes [32]. A partir de souches représentatives de chaque clade identifié auparavant, nous avons donc utilisé la séquence du gène codant pour la protéine VirB5 qui compose le pilus du SST4 VirB/D4 (impliquée dans l'invasion de la niche primaire) pour confirmer la spécificité d'hôte. Cependant la phylogénie basée sur le gène *virB5* n'a pas révélé la spécificité d'hôte observée en utilisant les gènes de ménage. Ce dernier résultat montre que l'interaction entre les espèces de *Bartonella* et leur hôte ne dépend pas uniquement de la protéine VirB5, mais d'un système bien plus complexe faisant intervenir une multitude de gènes codant pour des facteurs de virulence, tels que d'autres SST4, des adhésines, des autotransporteurs, ... En effet, la comparaison des génomes complets de quatre espèces de *Bartonella* (*B. bacilliformis* et *B. quintana* spécifiques de l'homme, *B. henselae* spécifique du chat et *B. tribocorum* spécifique du rat) a permis de découvrir que ces bactéries partagent 959 gènes codant pour des protéines impliquées dans l'invasion de l'hôte [45]. De surcroit, la

comparaison des séquences complètes des génomes d'espèces associées aux rongeurs (*B. tribocorum* et *B. grahamii*) avec ceux d'espèces inféodées au chat et à l'homme a révélé que ces espèces possèdent une quantité de gènes de virulence bien plus importante que les espèces spécifiques de l'homme et du chat [27].

Dans un troisième temps, en quantifiant les événements de recombinaison homologue au sein des souches de *B. taylorii* et de *B. grahamii* obtenues lors de cette étude, nous avons montré que la recombinaison joue un rôle important dans la diversification de ces souches. De plus, nous avons constaté que les événements de recombinaison au sein des souches de *B. taylorii* étaient beaucoup plus fréquents qu'au sein des souches apparentées à *B. grahamii*. Ces résultats sont cohérents avec la différence de diversité observée auparavant entre ces deux espèces. De façon étonnante, l'analyse du génome complet d'une souche de *B. grahamii* isolée en Suède a conduit à la découverte d'un agent de transfert de gène qui enveloppe des morceaux d'ADN bactérien de façon aléatoire [27]. Au regard de nos résultats, il est très probable que *B. taylorii* possède un mécanisme similaire. Le séquençage des génomes complets des souches de *B. taylorii* nous permettra de vérifier cette hypothèse.

Par ailleurs, en identifiant et en visualisant les événements de recombinaison homologue qui se sont produits entre les souches, nous avons montré que, malgré la barrière physique liée à la spécificité d'hôte, les souches de *B. taylorii* associées aux mulots sylvestres ont l'opportunité d'échanger du matériel génétique avec les souches de *B. taylorii* et de *B. grahamii* associées aux campagnols roussâtres. Ces transferts horizontaux entre les différentes souches témoignent donc de leur co-présence à un moment donné au cours de leur cycle de vie. Une étude américaine a révélé que presque toutes les puces, infestant une population de rats du genre *Sigmodon*, sont co-infectées par différents génotypes de *Bartonella* [46]. Nous avons donc supposé que les souches de *Bartonella* peuvent être transmises par le même vecteur arthropode, où elles auraient l'opportunité de recombiner. De plus, la communauté de puces infestant les campagnols roussâtres et les mulots sylvestres en forêt de Sénart a déjà été étudiée, et la diversité de puces s'est révélée équivalente entre les 2 espèces de rongeurs, appuyant l'hypothèse d'échanges génétiques au sein du vecteur [47]. Une fois de plus, l'étude de la diversité d'espèces de *Bartonella* au sein de la population de Siphonaptères infestant les campagnols

roussâtres et les mulots sylvestres devrait nous permettre de vérifier cette hypothèse.

Les 2 hôtes des bartonellae, l'hôte mammifère et le vecteur arthropode, influencent de façon majeure la structuration des souches de par la spécificité d'hôte et la compétence vis-à-vis des souches respectivement [5]. Cependant, d'autres facteurs peuvent impacter sur la diversité et la dynamique des souches de *Bartonella*. Au sein des populations naturelles de campagnols agrestes en Angleterre, il est maintenant avéré que *B. taylorii* interagit avec d'autres microorganismes, tels que le cowpox virus, *Babesia microtii* ou bien *Anaplasma phagocytophylum* [33]. Une multitude d'autres microorganismes peuvent infectés les rongeurs et donc potentiellement interagir avec les espèces de rongeurs au sein des populations naturelles. Une étude sur la prévalence et la diversité des espèces de *Borrelia burgdorferi* sensu lato, agent de la borréliose de Lyme, au sein d'une population de campagnols roussâtres a été menée dans la forêt de Sénart [34], ce qui nous a conduits à évaluer l'occurrence des co-infections par *Borrelia* et des souches de *Bartonella* parmi les campagnols pour ainsi vérifier si les 2 genres bactériens peuvent interagir. Lors de cette étude, qui constitue le troisième chapitre de cette thèse, nous avons montré que ces campagnols roussâtres sont infectés par divers génotypes affiliés à 3 espèces de *Bartonella* (*B. taylorii*, *B. grahamii* et *B. doshiae*) avec une prévalence de 56,4% et par un unique génotype de *Borrelia afzelii* présent dans 11% de la population. Nous avons constaté que les co-infections par *Borrelia afzelii* et les espèces de *Bartonella* peuvent se produire au sein de la population de campagnols mais semblent dues au hasard, suggérant que les 2 genres bactériens n'interagissent pas. Toutefois, la surveillance de plusieurs agents pathogènes (*Borrelia burgdorferi*, *Babesia microti* et *Ehrlichia* spp.) connus pour être véhiculés par les tiques au sein d'une population de souris à pattes blanches (*Peromyscus leucopus*) menée dans le centre ouest des Etats-Unis d'Amérique a permis la découverte de *B. vinsonii* subsp. *arupensis* [48]. Etonnement, les auteurs de cette étude ont remarqué que cette espèce de *Bartonella* infectait les souris à pattes blanches conjointement avec *Borrelia burgdorferi* [48]. Néanmoins, lors de cette étude en 1998, le complexe d'espèces *Borrelia burgdorferi* sensu lato n'était pas encore clairement subdivisé en espèces distinctes [35]. A l'heure actuelle, ce complexe d'espèces comprend 18 espèces structurées géographiquement, *Borrelia*

*afzelii* étant présente uniquement sur le continent eurasiatique [35]. Ces observations indiquent donc que les interactions entre les bartonellae et la communauté de parasites avec qui elles cohabitent au sein des populations de rongeurs varient selon la localisation des souches de *Bartonella*, leurs propriétés biologiques et celles des autres microorganismes, ainsi que la physiologie de l'hôte.

En outre, la diversité et la dynamique des espèces de *Bartonella* sont sujettes à d'autres facteurs liés à l'hôte et au vecteur. Afin d'étudier l'impact de l'abondance de l'hôte et des vecteurs, ou bien de la physiologie de l'hôte, plusieurs études longitudinales ont été réalisées en Afrique, aux Etats-Unis et en Europe. Au sein des populations de rongeurs, toutes ces études ont montré des variations saisonnières et liées à l'âge, des taux de prévalence des espèces de *Bartonella* [12, 49-54]. Par exemple, dans les états de l'est des Etats-Unis, des variations mensuelles de la prévalence des espèces de *Bartonella* au sein d'une population de rats du genre *Sigmodon* (*Sigmodon hispidus*) ont été observées, avec des taux allant de 49% en juin jusqu'à 95% en octobre [50]. De plus, parmi les individus de cette population, les auteurs ont montré que 90% des jeunes individus étaient infectés contre un peu moins de 40% chez les individus les plus âgés [50]. Les variations saisonnières de prévalence observées en Europe et aux Etats-Unis sont probablement liées aux variations saisonnières des réponses inflammatoires des hôtes dues à la photopériode et à la période de reproduction [55], alors que les variations liées à l'âge des individus seraient dues à l'acquisition d'une immunité contre les souches de *Bartonella*. Toutes les études précédentes ont décrit ces variations de prévalence au niveau de l'espèce. Les rongeurs choisis lors de ce projet ont été préalablement utilisés pour étudier les variations de densité des populations de campagnols et de mulots en forêt de Sénart. Ces rongeurs ont donc été capturés tous les mois, de mars à novembre en 2007 et 2008. De façon étonnante, les densités de campagnols et de mulots présentent une évolution identique, avec des variations très marquées entre 2007 et 2008. Afin d'observer l'effet de la densité des populations de rongeurs sur la diversité et la prévalence des souches de *Bartonella*, nous avons l'intention d'étudier les variations saisonnières des souches de *Bartonella*, précisément identifiées, pour les mettre en relation avec les variations de densité des campagnols et des mulots.

Lors de ce travail de thèse, nous avons isolé et caractérisé une collection unique de souches de *Bartonella* obtenue à partir d'une population naturelle de rongeurs sylvatiques. Par une identification précise de ces souches, nous avons démontré une forte association des génotypes avec les espèces de rongeurs. Cependant, les bases génétiques de cette spécificité d'hôte restent encore à découvrir. Le séquençage et la comparaison des génomes complets de souches représentatives de *B. doshiae*, de *B. grahamii* et de chaque clade de *B. taylorii* nous permettront d'identifier et de mieux comprendre les mécanismes moléculaires impliqués dans la spécificité d'hôte ainsi que les différences subtiles qui peuvent exister entre des souches phylogénétiquement proches mais qui infectent des hôtes différents.



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# Annexe I

## First detection of the novel human pathogen “*Candidatus Neoehrlichia mikurensis*” in wild rodents, France.

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For the first time, DNA from human pathogenic "*Candidatus* Neoehrlichia mikurensis" has been detected in rodents trapped in France.

Emerging infectious diseases are a significant burden on public health. A recent study analyzing a database of 335 such disease events, indicated that they are dominated by zoonoses (accounting for 60%). The majority of these zoonoses (71.8%), originate in wildlife and are increasing significantly over time (1). Interestingly, half of these emerging infectious events are caused by bacteria which mostly belong to the proteobacterial order of rickettsiales (1). This study thus reveals the substantial risk of wildlife zoonotic and vector-borne diseases to public health.

In Western Europe, the most common vector for human and animal pathogens is the widespread and abundant tick, *Ixodes ricinus*, which also happens to be a major vector of pathogens responsible for rodent-borne disease (2). Lyme borreliosis, caused by the bacteria *Borrelia burgdorferi* s.l. is the most prevalent tick and rodent-borne illness. In addition to Lyme disease, patients bitten by ticks can also be infected by other bacteria belonging to three main genera: *Anaplasma* sp., *Bartonella* sp. and *Rickettsiae* sp. These bacterial species have all been associated with human tick-borne infections over the last 10 years (2). Furthermore, during the last decade, DNA of a new species of intracellular bacteria belonging to the Anaplasmataceae family, was sequenced from ticks and rodents originating from Europe and Asia (3-5). The first isolate of this organism was obtained in 2004 from wild rats *Rattus norvegicus* and *Ixodes ovatus* from Japan (6). The morphological and molecular characterization of the isolate in comparison to earlier closely related GenBank® sequences, supported a novel genetic cluster within the family Anaplasmataceae. Consequently, the nomenclature “*Candidatus Neohrlichia mikurensis*” was proposed for all current organisms in this group (6). Since its discovery in 2004, it has been identified in different tick species, including *Ixodes ricinus* in Europe, and in other small rodents suspected to be the bacterium’s natural main reservoir (7-12). The first case of human “*Candidatus N. mikurensis*” infection was reported in 2010 in a Swedish patient (13). In that same year, five other human infections were also described in Germany, Switzerland and the Czech Republic (14). More recently, a canine infection due to this bacteria was reported in Germany (15). Symptoms described in all cases were general and non specific (fever, cough, anemia, headache, pulmonary infiltrate, malaise, myalgia, joint pain, extreme fatigue, skin erythema ...) making diagnosis difficult, particularly in the absence



of serological tests. The actual incidence of human “*Candidatus N. mikurensis*” infection is thus likely to be much higher than is currently reported in Europe. In addition, these studies all advocated an assessment of the occurrence of this micro-organism in those countries in which it has not yet been evaluated.

In this study we demonstrate for the first time the presence of “*Candidatus N. mikurensis*” in France, specifically in bank vole, *Myodes glareolus*, one of its suspected reservoirs.

### **The study**

During the course of a 2008 study into the epidemiology of *Puumala* hantavirus, voles were trapped in the French Ardennes along a transect line of approximately 80 km (16). Along this transect, we sampled six sites in forested areas and four sites in fragmented habitats (i.e. hedge networks). Animals were sacrificed by cervical dislocation, then weighed, sexed and dissected. Spleens were placed in RNAlater® (Sigma-Aldrich, MO, USA) and stored at -20°C for further analyses. Genomic DNA was extracted from a portion of spleen using the DNeasy kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions, into a final elution volume of 100 µL.

The presence of DNA from “*Candidatus N. mikurensis*” in rodent spleen DNA was tested by PCR using specific primers targeting the “*Candidatus N. mikurensis*” *groEL* gene as previously described (15). In total, “*Candidatus N. mikurensis*” DNA was detected in 5/276 bank voles (1.8%). This prevalence lies within the range found in small rodents trapped in other European countries (from 0% to 11.5%) (9, 12). Positive rodents were trapped in forested patches in Woiries (49.903N; 4.763E), Elan (49.655N; 4.770E) and Croix-aux-Bois (49.419N; 4.824E) and also in a hedge network in Cliron (49.802N; 4.619E), where the tick *Ixodes ricinus* is also present in abundance (E. Ferquel, personal communication). Interestingly, all positive rodents were trapped in close proximity to human settlements and villages (from a distance of 300 m to 1 km).

All PCR products (a 1024 bp-fragment of the *groEL* gene) were then sequenced in forward and reverse directions by Eurofins MWG Operon (Germany). Sequences were

aligned and analysed for phylogetic purposes using SeaView v.4. (17). All sequences were identical and matched the "*Candidatus N. mikurensis*" *groEL* gene. The highest identity percentages obtained corresponded to the sequences of "*Candidatus N. mikurensis*" *groEL* genes from European cases, in particular: 99% nucleotide identity to sequences obtained from two human patients residing in Germany (accession no. EU810407.1 and EU810406.1) and to sequences obtained from a sick dog from Germany (accession no. EU432375). Lowest identity percentages corresponded to sequences from wild rodents from China (95% identity, accession no. JQ359066, JQ359067 JQ359068), to wild rodents and *Ixodes ovatus* from Japan (94% identity, accession no. AB074461, AB084583) and to raccoons from the United States (91% identity, accession no. EF633745).

The phylogeny was constructed using the neighbor-joining (BioNJ) method and a Kimura 2-parameter distance. Bootstrap analysis was carried out on 1,000 replicates. Phylogenetic analysis of the comparatively long *groEL* gene sequence showed highly significant clustering of the samples into three groups, suggesting the existence of at least three types of "*Candidatus N. mikurensis*" sequence variants (Figure 1). Clustering was not related to the host nor to the geographic sample origin. Sequences obtained from French bank voles all clustered together with sequences that had been amplified from sick human or animal samples from Europe.

## Conclusion

In France, *Candidatus N. mikurensis* is now established to be present in small rodents, the main source of blood meals for the larvae and nymphs of *I. ricinus*. Although human or animal infection has not yet been reported in France, positive rodents trapped in this study carried the same *Candidatus N. mikurensis* genotype as those bacterium found to cause disease in humans and animals. Furthermore, the positive rodents were located in close proximity to human dwellings, suggesting that *Candidatus N. mikurensis* is very likely to pose risks to public health in France. It is also interesting to note that the particular geographical region studied is endemic for nephropatia epidemica, whose mild symptoms, (body aches, chills, sweats, fatigue and high fever) could be confused

with those caused by *Candidatus N. mikurensis*. To date, diagnosis of *Candidatus N. mikurensis* infection has relied only on PCR amplification of the bacterial DNA. Therefore, the absence of serological tests combined with medical authorities' lack of knowledge relating to these bacteria, makes diagnosis particularly difficult. Thorough surveillance and improved diagnostic tools are required to provide more insight into the relevance of *Candidatus N. mikurensis* to public health.

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**Biographical sketch of first author:** Dr Muriel Vayssier-Taussat is the head of a research team at INRA. Her areas of interest include tick borne bacteria, her research being devoted to the development of molecular tools dedicated to identifying pathogens in ticks or in animals, and to elucidate their mechanisms of infection.

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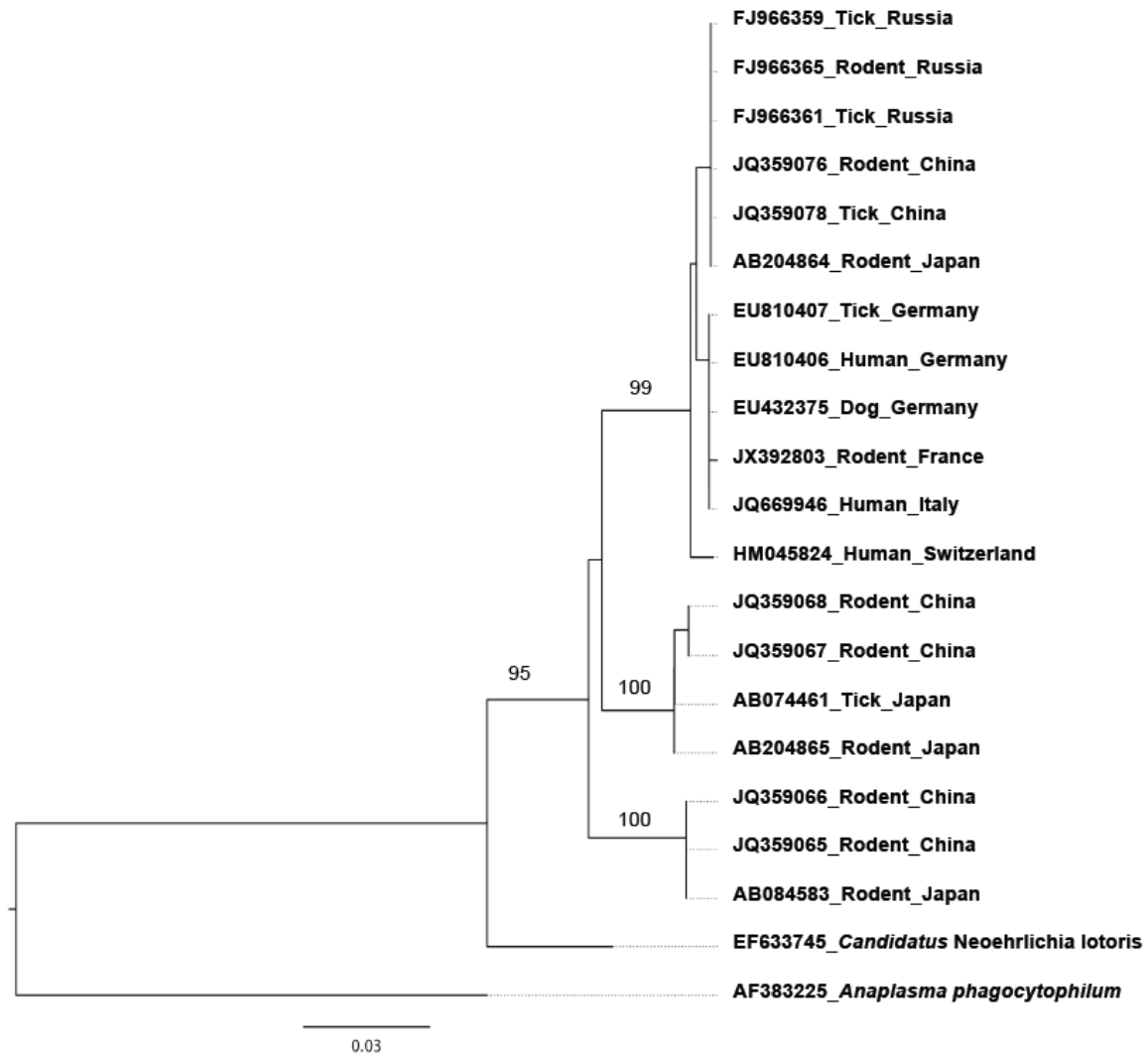
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**Figure legend:**

Figure 1: : Phylogenetic relationships between the unique "*Candidatus N. mikurensis*" genotype detected in a population of bank voles from the French Ardennes and other "*Candidatus N. mikurensis*" genotypes from other geographical regions, based on the sequence of the *groEL* gene. The phylogenetic tree was constructed by the neighbor-joining method using a Kimura 2-parameter distance. Bootstrap analysis was carried out on 1,000 replicates; values are indicated at the nodes. GenBank® accession numbers are indicated for each sequence used. The *groEL* sequence of *Anaplasma phagocytophilum* (accession n° AF383225) was chosen as an outgroup in the phylogenetic tree.





## Annexe II

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### Human pathogens in *Ixodes ricinus* ticks from Romania

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1           The tick *Ixodes ricinus* is a predominant vector of a large variety of pathogens of  
2 veterinary and medical importance in Europe (1). The most prevalent *I. ricinus*-borne  
3 infection in Europeans is Lyme borreliosis, a multisystemic disorder caused by  
4 spirochetes of the *Borrelia burgdorferi* sensu lato complex (2). In addition to *Borrelia*  
5 spp, individuals bitten by ticks can also be infected with many other pathogens, such as  
6 bacteria (*Anaplasma* spp., *Francisella* spp., *Coxiella burnetii*, *Bartonella* spp.,  
7 *Rickettsiae* spp. and *Neorhlichia mikurensis*), parasites (*Babesia* spp., *Theileria* spp.)  
8 and arboviruses (TBE, CCHF, EYA ...) (1). Symptoms induced by such pathogens are  
9 often diverse and non-specific, complicating accurate diagnosis of the disease.

10           In Romania, cases of Lyme disease and tick borne encephalitis (due to TBEV)  
11 have been identified (3). However, little is known about the public health impact of these  
12 diseases, and none of the other tick-borne pathogens present in Europe have been  
13 reported as detected in Romania. As *I. ricinus* is the most abundant and widespread tick  
14 in Romania (3), the impact of tick-borne disease is very likely to be underestimated.  
15 Therefore, the first step in evaluating the distribution of these potential pathogens is to  
16 establish their presence in ticks from previously unexplored areas. We thus undertook a  
17 study aiming to identify the main tick-borne bacterial and parasitic human pathogens  
18 known to be present in Europe but never previously detected in Romania. The presence  
19 of DNA from the spotted fever group (SFG)-*Rickettsia* spp., *Anaplasma* spp.,  
20 *Francisella tularensis*, and *Babesia* spp. was thus tested by PCR using specific primers  
21 for each pathogen or group of pathogens, as previously described (4) in 147 *I. ricinus*  
22 collected from roe deer and goats in two sites in Eastern Romania (Bacau (46°35 '0"N  
23 26°55 '0"E) and Galati (45°26 '22"N 28°2 '4"E)). Sequences obtained from Eurofins MGW  
24 Operon (Germany) were identified using BLAST from the National Center for  
25 Biotechnology Information ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) and compared with  
26 sequences available in GenBank.

27           DNA from *Rickettsia* spp. was detected in 20 ticks (13.6%). Sequence analyses  
28 revealed that 9 sequences (6.1%) were related to *R. monacensis* strain IRd/Serbia *gltA*  
29 gene, with 99-100% nucleotide similarity (accession no. GQ925820) and 11 (7.48%)  
30 were related to *R. helvetica gltA* gene with 99-100% nucleotide similarity (accession no.  
31 AM418450). DNA from *Anaplasma* spp. was identified in 33 ticks (22.4%). Analysis

1 revealed that 30 of the 33 amplified fragments showed 100% identity to the 16S rDNA  
2 gene of a symbiont in the *Anaplasmataceae* family, *Candidatus Midichloria mitochondrii*  
3 (accession no. EU780455), and the remaining three were related to known pathogenic  
4 species identified here for the first time in Romania: two (1.4%) exhibited 100% identity  
5 to *A. phagocytophilum* (accession no. EU982548) and one (0.7%), showed 99%  
6 similarity to *Ehrlichia muris* (accession no. GU358691). *F. tularensis* specific DNA was  
7 amplified from four DNA extracts (2.7%). All four sequences were identical and shared  
8 99% similarity with the *F. tularensis* peptidyl-propyl cis-trans isomerase gene (accession  
9 no. CP003048). *Babesia* spp. specific DNA was amplified in only one DNA extract  
10 (0.7%), and shared 99% sequence identity with the *Babesia* sp. EU1 18S rDNA gene  
11 (accession no. HQ830266).

12 This is the first report of the identification of the human pathogens *R.*  
13 *monacensis*, *R. helvetica*, *A. phagocytophilum*, *E. muris* and *Babesia* EU1 in Romania.  
14 *R. monacensis* (also known as the Cadiz agent or *Rickettsia* IRS3 and IRS4), was first  
15 identified in *I. ricinus* samples from Germany (5) and was recently recognized as the  
16 cause of Mediterranean spotted fever-like rickettsiosis in Spain (6). *R. helvetica* was  
17 isolated in Switzerland from *I. ricinus* in 1979 and since then in many other European  
18 countries (7), and has been associated with human infections in the late 90s (8). The  
19 role of animals as reservoirs for these pathogens is currently unknown. *A.*  
20 *phagocytophilum* is the causative agent of granulocytic anaplasmosis in humans, cattle,  
21 horses and dogs, and is widespread throughout Europe (8). *E. muris* has been detected  
22 in ticks from Finland, European Russia and in both ticks and rodents from Slovakia. In  
23 2009 it was also detected in patients suffering from febrile illness in the USA (9).  
24 *Babesia* sp. EU1 has been detected in roe deer and ticks in several countries and in  
25 2003 was associated with human disease for the first time in Italy and Austria (4, 10).  
26 As stated for the other pathogens, this is the first description of its presence in Romania.  
27 Concerning tularemia, this disease is known to be present in Romania, and is thought to  
28 be exclusively and directly transmitted by hares. However, the detection of *F. tularensis*  
29 DNA in *I. ricinus* suggests that this bacterium might also be tick-bourne. In conclusion,  
30 detecting the DNA of these human pathogens in ticks strongly suggests that these  
31 microorganisms circulate in the country. As they can all affect humans, our study

- 1 highlights the urgent requirement for further research to assess their impact on public
- 2 health in Romania.
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2 This study was partially funded by the EU grant FP7-261504 EDENext and is catalogued by the  
3 EDENext Steering Committee as EDENext 043 (<http://www.edenext.eu>). The contents of this  
4 publication are the sole responsibility of the authors and do not necessarily reflect the views of  
5 the European Commission. We also thank the group Tiques et Maladies à Tiques group (TMT)  
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## RESUME

Les bactéries du genre *Bartonella* sont des alpha-proteobacteria intracellulaires facultatives, capables d'infecter les érythrocytes d'une multitude d'espèces de mammifères. A ce jour, 33 espèces de *Bartonella* ont été recensées incluant une vingtaine d'espèces identifiées chez des rongeurs, dont certaines sont associées à des maladies chez l'homme. Des études provenant de différentes parties du monde montrent que les populations de rongeurs hébergent une grande diversité d'espèces de *Bartonella* avec des taux de prévalence élevés. Plusieurs facteurs liés à l'hôte, au vecteur et à l'environnement dans lequel ils évoluent influencent et auraient, par le passé façonné cette diversité. L'objectif de cette thèse a été d'élucider certains de ces facteurs en étudiant la diversité des souches de *Bartonella* circulant au sein d'une population de rongeurs sylvatiques dans une zone de forêt périurbaine française, la forêt de Sénart. Dans un premier temps, en nous basant sur une phylogénie précise et robuste, nous avons identifié 63 génotypes apparentés à 3 espèces connues de *Bartonella* (*B. taylorii*, *B. grahamii* et *B. doshiae*) communément retrouvées au sein des rongeurs sylvatiques en Europe et à une nouvelle espèce proche de *B. rochalimae*. Nous avons montré que la majorité des souches obtenues lors de cette étude sont fortement associées soit aux campagnols roussâtres soit aux mulots sylvestres. Malgré la barrière physique liée à la spécificité d'hôte, nous avons observé des échanges de matériel génétique entre les souches de *Bartonella* associées aux campagnols roussâtres et les souches adaptées aux mulots, suggérant que ces souches peuvent cohabiter au sein de leur vecteur. Dans un second temps, en évaluant l'impact de l'espèce bactérienne *Borrelia afzelii* sur la présence et la diversité des souches de *Bartonella* au sein d'une population de campagnols roussâtres par des outils statistiques, nous avons observé des co-infections par les deux genres bactériens au sein d'un même rongeur, mais ces deux bactéries ne semble pas interagir.

## ABSTRACT

Gram-negative bacteria belonging to the genus *Bartonella* are facultative intracellular bacteria which can infect the red blood cells of a wide variety of mammal species. To date, 33 *Bartonella* have been validly described including 20 of them identified in rodents, of which some are associated with human case of illness. Studies from many parts of the world indicate that rodent populations carry high diversity of *Bartonella* species with high rates of prevalence. Several factors linked to host, arthropod vector and the environment in which they have evolved have shaped and influence this diversity. The purpose of this PhD project was to better understand some of these factors by studying the *Bartonella* strains circulating within a community of sylvatic rodents in a French suburban forest, the Sénart forest. Firstly, based on an accurate and robust phylogeny, we identified 63 *Bartonella* genotypes affiliated to 3 known *Bartonella* species (*B. taylorii*, *B. grahamii* and *B. doshiae*), frequently recovered from woodland rodents in Europe, and to one new *Bartonella* species in proximity to *B. rochalimae*. We demonstrated that the majority of genotypes obtained here are highly specific either bank voles or wood mice. Despite the physical barrier created by host specificity, we observed exchanges of genetic material between *Bartonella* strains associated to bank voles and strains specific to wood mice, suggesting that these strains can co-habit within the arthropod vector. Secondly, we assessed, using statistical tools, the impact of *Borrelia afzelii* on prevalence and diversity of *Bartonella* strains within a population of field voles, and we observed that these two bacteria can be found in conjunction, but they do not appear to interact.

**Mots-clés:** *Bartonella*, diversité, phylogénie, spécificité d'hôte, recombinaison homologue, *Borrelia afzelii*, interaction