

1 **Title:** The importance of lizards and small mammals as reservoirs for *Borrelia*  
2 *lusitaniae* in Portugal

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29 **Running title:** *B. lusitaniae* in lizards and small mammals in Portugal

30

31

32 **Summary**

33 *Borrelia lusitaniae* is a pathogen frequent in the Mediterranean area. Apart from lizards,  
34 evidence for birds and small mammals as competent reservoirs for this genospecies has  
35 been occasional. We collected questing ticks, skin biopsies and *Ixodes* sp. ticks feeding  
36 on lizards, birds and small mammals in a *B. burgdorferi* s.l. enzootic area to assess their  
37 importance in the maintenance of *B. lusitaniae*. *B. lusitaniae* was the most prevalent  
38 genospecies in questing ticks and was commonly found in larvae feeding on  
39 *Psammodromus algirus*. One biopsy infected with *B. lusitaniae* was collected from the  
40 tail of one *Podarcis hispanica*, which suggests systemic infection. *I. ricinus* larvae  
41 feeding on *Apodemus sylvaticus* were infected with *B. lusitaniae* but with a lower  
42 prevalence. Our results reinforce the importance of lizards as reservoirs for *B.*  
43 *lusitaniae*, suggesting that *P. algirus*, in particular, acts as main reservoir for *B.*  
44 *lusitaniae* in Portugal.

45

## 46 **Introduction**

47

48 *Borrelia lusitaniae* is a genospecies frequent in the Mediterranean area with  
49 focal distribution in central and northern Europe. In Portugal, this is the most frequent  
50 genospecies infecting questing ticks (Baptista, 2006; Lopes de Carvalho et al., 2008a).  
51 This is also the case in Tapada de Mafra, a mixed deciduous forest, where *Ixodes*  
52 *ricinus* ticks are abundant and are frequently infected with *Borrelia burgdorferi* s.l.,  
53 with a higher prevalence of *B. lusitaniae* when compared to other genospecies (Norte et  
54 al., 2013a). *B. lusitaniae* reservoir hosts have not been studied in detail, apart from  
55 reptiles, which are confirmed reservoirs through xenodiagnosis (Dsouli et al., 2006).  
56 Some observations have suggested that both small mammals (Lopes de Carvalho et al.,  
57 2010) and birds (Poupon et al., 2006) can also act as reservoirs for this genospecies but  
58 further support is needed. In one previous survey at Tapada de Mafra (see Norte et al.  
59 2012), where *B. lusitaniae* was the most prevalent genospecies in questing ticks, we  
60 found no evidence of reservoir competency of avian hosts for *B. lusitaniae* (Norte et al.,  
61 2013b). This is a pathogenic genospecies (da Franca et al., 2005; Lopes de Carvalho et  
62 al., 2008b), and it is important to better understand which factors contribute to its  
63 maintenance in nature, to eventually minimize infection and disease risk. In this study,  
64 we collected samples (feeding ticks and tissues) from mammals, birds and lizards, and  
65 questing ticks, to assess the potential of each of these vertebrate groups as reservoir for  
66 *B. lusitaniae*, and elucidate which species contributes the most to the maintenance of *B.*  
67 *lusitaniae* in Tapada de Mafra enzootic focus..

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69

## 70 **Results and discussion**

71

72 *B. burgdorferi s.l infection in questing ticks*

73

74 A total of 749 *Ixodes* spp. were collected from the vegetation, including 748 *I. ricinus*  
75 and one *I. frontalis* nymph (Table 1). From these, 373 were tested for *B. burgdorferi* s.l.  
76 infection using a nested PCR targeting the 5S-23S rDNA intergenic spacer region  
77 (Rijpkema et al., 1995; Supplemental Material 1). *B. burgdorferi* s.l. prevalence in  
78 questing *I. ricinus* was 0% in larvae (0/100), 5.3% (14/262) in nymphs and 16.7% (1/6)  
79 in adults (Table 1). As expected, *B. burgdorferi* s.l. prevalence increased from larval to  
80 adult stage of *I. ricinus* ticks, which is in support of the low transovarial transmission of  
81 this agent in this tick species (Bellet-Edimo et al., 2005), and increasing number of  
82 hosts on which the ticks fed along their life stages. Therefore, detection of infection in *I.*  
83 *ricinus* larvae feeding on a host strongly suggests acquisition from the host (except in  
84 cases of co-feeding; Randolph et al., 1996). The following genospecies were detected in  
85 questing ticks: *B. lusitaniae*, *B. valaisiana*, *B. garinii* and *B. afzelii* (Table 1). The most  
86 prevalent genospecies was *B. lusitaniae*, infecting 3.0% (8/262) of the *I. ricinus* nymphs  
87 (Table 1), which is in agreement with previous findings for the same area (Baptista,  
88 2006; Norte et al., 2013b). The finding of one *I. frontalis* nymph questing in the  
89 vegetation is worth highlighting because collections of this tick species by flagging are  
90 rare (Pérez-Eid, 2007; Bona and Stanko, 2013).

91

92 *B. burgdorferi s.l infection in ticks and tissues from vertebrates*

93

94 Lizards

95

96 Fifty lizards, including 2 recaptures, belonging to 4 species were sampled (Table  
97 2). From these, 426 *I. ricinus* (379 larvae and 47 nymphs) were collected (Table 2). 346  
98 *I. ricinus* were tested for *B. burgdorferi* s.l. infection and the prevalence was 13.3%  
99 (46/346; Table 2), with all the infections belonging to *B. lusitaniae*. Infected ticks were  
100 feeding on *Psammodromus algirus*, *Podarcis hispanica* and *Lacerta shreiberi*.  
101 However, from these lizard species, only *P. algirus* carried *B. lusitaniae* infected larvae  
102 and some of those individuals were infested only by larvae. This suggests reservoir  
103 competence of this lizard species for *B. lusitaniae*. *P. algirus* had already been  
104 confirmed as reservoir for *B. lusitaniae* in Tunisia by xenodiagnosis (Dsouli et al.,  
105 2006), and additional evidence of the role of lizard species as reservoirs for *B. lusitaniae*  
106 has been adding up (Richter and Matuschka, 2006; Foldvari et al., 2009; De Sousa et  
107 al., 2012).

108 From the 47 tail biopsies collected (42 in duplicate: one for inoculation into  
109 Barbour-Stoenner-Kelly complete (BSK II) medium, and the other for direct DNA  
110 extraction – see Supplemental Material 1), in one biopsy from *P. hispanica*, *B.*  
111 *lusitaniae* DNA was detected, suggesting systemic infection. *B. burgdorferi* s.l.  
112 detection in biopsies was rather low (in lizards and other vertebrates groups, see  
113 below). In the case of lizards, this may be attributed to the fact that the tissue from the  
114 lizards' tail is not the best for *Borrelia* detection, when compared to e.g. the collar  
115 tissue. Majláthová et al. (2006) collected both tail samples and collar skin biopsies from  
116 *Lacerta viridis* but only detected *B. burgdorferi* s.l. in the collar biopsies (18.6%).  
117 Foldvari et al. (2009) collected both collar scales and toe clips from lizards, and the  
118 percentage of infected tissues was higher for collar scales (8.2%) than for toe clips  
119 (2.0%). However, detection from collar scales may reflect local infection, because most  
120 of the feeding ticks are attached in this area, rather than a systemic infection. *B.*

121 *burgdorferi* s.l. has been detected in lizard tail tissues with success in other studies  
122 (Amore et al., 2007; De Sousa et al. 2012). Another potential difficulty in obtaining  
123 isolates from field derived inoculations in BSK-II medium arises from the susceptibility  
124 of these cultures to contamination. Cultures contaminated soon after inoculation and  
125 this may have inhibited *Borrelia* growth and/ or the PCR on the DNA extracted from  
126 these cultures.

127         The high *I. ricinus* larval tick infestation intensity in *P. algirus* (Table 2), than in  
128 other sampled vertebrate species (see bellow), suggests the great potential of this  
129 species to infect a large number of ticks in this area, acting as the source of *B. lusitaniae*  
130 infection to questing nymphs in the area. The other captured lizards in our study (*P.*  
131 *hispanica*, *L. shreiberi* and *Timon lepidus*, formerly *Lacerta lepida*) do not seem to have  
132 such an important role as reservoirs for *B. lusitaniae* as *P. algirus*, because either they  
133 were not infested by ticks or the infection prevalence in their infesting larvae was zero.  
134 Although the number of *Lacerta* sp. individuals captured was low, precluding a reliable  
135 evaluation of their reservoir status, the number of *P. hispanica* sampled individuals was  
136 relatively high, and the lack of infection in its large number of infesting larval *I. ricinus*  
137 contrasts with the finding of one *B. lusitaniae* positive biopsy collected from this lizard  
138 species. The reservoir competence for a *Borrelia* genospecies may vary between related  
139 species occurring sympatrically, and which are both important tick hosts (e.g *P. algirus*  
140 and *P. hispanica*). Although the reservoir competence of lizards for *B. burgdorferi* s.l.  
141 may be species' specific, the range of lizard species for which evidence as reservoirs for  
142 *B. lusitaniae* has been compiled is quite large and includes: *L. viridis*, *Podarcis muralis*,  
143 *Podarcis taurica* and *Lacerta agilis* in central Europe (Richter and Matuschka, 2006;  
144 Majláthová et al., 2006; Foldvari et al., 2009), *P. muralis* in Tuscany (Amore et al.,

145 2007), and *Teira dugesii* in Madeira's island, Portugal (De Sousa et al., 2012). More  
146 studies are needed to confirm the reservoir potential of *P. hispanica* for *B. lusitaniae*.

147

#### 148 Mammals

149

150         Seventy-eight small mammals belonging to 5 species were captured, including  
151 30 recaptures. The most common species captured was the wood mouse *Apodemus*  
152 *sylvaticus*. Forty-one small mammals were infested by ticks, and 239 *Ixodes* spp. ticks  
153 were collected from them, including 234 *I. ricinus* in larval stage (Table 3). 226 *Ixodes*  
154 spp. ticks were screened for *Borrelia* infection, whose prevalence was 0.9% (2/226). All  
155 infections corresponded to *B. lusitaniae* and they were detected in *I. ricinus* larvae  
156 feeding on an *A. sylvaticus*. Those infected ticks were derived from the same animal,  
157 infested only by larvae. This finding supports previous results that *A. sylvaticus* acts as  
158 competent reservoir for *B. lusitaniae* (Lopes de Carvalho et al. 2010). The percentage of  
159 *B. lusitaniae* infected larvae feeding on *A. sylvaticus* was much lower than those feeding  
160 on *P. algirus*, suggesting a less prominent role of this small mammal as amplification  
161 agent of this genospecies in the study area. However, the number of small mammal  
162 recaptures was quite high (30/78; 38.5%). Because they were not individually marked,  
163 *Borrelia* prevalence in ticks derived from small mammals could have been  
164 underestimated if spirochete-free individuals were captured significantly more often  
165 than others.

166         Forty-six ear biopsies were collected (38 in duplicate) from 36 *A. sylvaticus*, 7  
167 *Mus* spp., 2 *Microtus lusitanicus* and 1 *Crossidura russula*. This tissue has been used  
168 previously with success in direct detections and to obtain *B. burgdorferi* s.l. isolated  
169 cultures (Sinsky and Piesman, 1989; Hanincová et al., 2003a), but none of the collected

170 biopsies in this study were found infected (either through culture on BSK II medium or  
171 direct DNA extraction). One out of the two cultures from the heart of *A. sylvaticus* was  
172 positive, but the genospecies was impossible to determine. None of the two bladder  
173 derived cultures from the same individuals were positive.

174         Additionally 1 (larva) and 11 (9 nymphs and 2 adult females) *I. ricinus* were  
175 collected from a rabbit and a wild boar, respectively, but were negative for *B.*  
176 *burgdorferi* s.l. Two biopsies were also taken from the ear of the rabbit but were  
177 negative for *B. burgdorferi* s.l., both through culture and direct DNA extraction.

178         We did not detect *B. afzelii* either in ticks feeding on any of the sampled hosts or  
179 their tissues. Although the prevalence of this genospecies was relatively low in questing  
180 nymphs, it was unexpected that ticks collected feeding on potential *B. afzelii* reservoirs  
181 hosts, such as small mammal species (Hanincová et al., 2003a; Cadenas et al., 2007)  
182 and wild boars, were negative. In North Eastern Europe, *I. ricinus* larvae feeding on  
183 *Lacerta* sp. were infected with *B. afzelii* (Majláthová et al., 2006; Foldvari et al., 2009),  
184 but our sampled lizard species did not harbour *B. afzelii* positive ticks. Possibly other  
185 mammal species which were not sampled, such as hedgehogs (Skuballa et al., 2012),  
186 foxes or some Artiodactyl species (Cadenas et al. 2007) act as *B. afzelii* reservoirs in  
187 this area.

188

## 189 Birds

190

191         One-hundred and eight birds were captured, including 5 recaptures, belonging to  
192 22 species, and 31 were infested with ticks: 243 *Ixodes* spp. ticks were collected,  
193 including 236 *I. ricinus* (204 larvae and 32 nymphs; Supplemental Material 2). We  
194 screened 220 *Ixodes* sp. for *B. burgdorferi* s.l. infection. Only *Turdus merula* (3 out of 5



195 birds infested) harboured infected ticks, and the genospecies detected were *B. garinii*,  
196 the most prevalent genospecies (16/97; 16.5%), followed by *B. valaisiana* (10/97;  
197 10.3%) and *B. turdi* (3/97; 3.1%; Table 4). A mixed infection by these three  
198 genospecies was detected in one *I. ricinus* larva through a Reverse Line Blot (Gil 2005)  
199 using specific probes described previously (Ripkema 1995, Gil 2005, Gern 2010; see  
200 Supplemental Material 1). The relatively high prevalence of *B. garinii* and *B. valaisiana*  
201 in ticks derived from *T. merula* is consistent with previous findings from the same area  
202 and, with other studies which found that birds from the genus *Turdus* are competent  
203 reservoirs for these genospecies (Taragel'ová et al., 2008; Norte et al., 2013a; Norte et  
204 al., 2013b). *B. turdi*, detected in a lower prevalence, is a genospecies associated with  
205 bird-reservoirs (Norte et al., 2013a,b).

206         Twenty-four biopsies were collected in duplicate from 24 birds which  
207 corresponded to 77% (24/31) of the birds which carried ticks, belonging to 10 species  
208 (Supplemental Material 2), but all were negative for *B. burgdorferi* s.l.

209

#### 210 *Genetic variability of B. lusitaniae strains in circulation at Tapada de Mafra*

211

212 In this study, we detected 3 genetic variants of the analysed partial 5S-23S intergenic  
213 spacer region (from the positions 254 to 453). The sequences were aligned using  
214 Multalin software (Corpet, 1988) and compared with other published sequences. Two  
215 variants were reported before in questing ticks from the same area (Norte et al., 2013b)  
216 but a new one was found in *I. ricinus* larvae and nymphs feeding on *P. algirus* and in *I.*  
217 *ricinus* larvae feeding on *A. sylvaticus* (PoTiBlus4). This variant differed between 3 and  
218 9 base pairs from the other genetic variants reported from the same area (PoTiBlus1,  
219 PoTiBlus2, PoTiBlus3, PoTiBmf364 and PoTiBl37; Norte et al. 2013b).

220

221           The results of this study are in partial agreement with those of Amore et al.,  
222 (2007) in Tuscany, where *B. lusitaniae* was the most prevalent *Borrelia* genospecies in  
223 questing ticks and the hosts harbouring *B. lusitaniae* infected ticks were lizards (in their  
224 case *Podarcis* sp.). Amore et al. (2007) also found that 19-25% of the blood and biopsy  
225 samples from *Podarcis* sp. were positive for this genospecies, but, neither mice nor  
226 passerine birds were infected or harboured *B. lusitaniae* infected ticks. This shows that  
227 host specificity for *Borrelia* genospecies may vary with *Borrelia* subtypes, different tick  
228 vector species/races, and host composition in different geographic areas differing in  
229 habitat structure and biocenosis (Gern, 2008). Therefore, specific and localised studies  
230 are needed in each enzootic area to properly understand the ecology of this zoonosis.

231

#### 232 **Nucleotide sequence accession numbers**

233 *B. lusitaniae* rrf (5S)-rrl (23S) intergenic spacer region nucleotide sequences obtained in  
234 this study, and in Norte et al. (2013b) study from the same area, have been deposited in  
235 GenBank with the accession numbers: KJ857486 (PoTiBlus2) and KJ857487  
236 (PoTiBlus4).

237

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245

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343 **Table 1.** *Borrelia burgdorferi* s.l. infection prevalence in *Ixodes* sp. ticks collected  
 344 questing in the vegetation at Tapada de Mafra from February to June 2013.

| Species                 | stage | sex | no.<br>collected | no.<br>tested | no. <i>Bbsl</i><br>positive ticks<br>(%) | No. of ticks infected with <i>Bbsl</i> genospecies<br>(%) |          |         |         |
|-------------------------|-------|-----|------------------|---------------|--|---|----------|---------|---------|
|                         |       |     |                  |               |  | LU  | VA       | GA      | AF      |
| <i>Ixodes ricinus</i>   | L     |     | 371              | 100           | 0 (0)                                    | 0 (0)   | 0 (0)    | 0 (0)   | 0 (0)   |
|                         | N     |     | 367              | 262           | 14 (5.3)                                 | 8 (3.0)   | 3 (1.1)  | 2 (0.8) | 1 (0.4) |
|                         | A     | M   | 6                | 6             | 1 (16.7)                                 | 0 (0)   | 1 (16.7) | 0 (0)   | 0 (0)   |
|                         |       | F   | 4                | 4             | 0/4 (0)                                  | 0 (0)   | 0 (0)    | 0 (0)   | 0 (0)   |
| <i>Ixodes frontalis</i> | N     |     | 1                | 1             | 0/1 (0)                                  | 0 (0)   | 0 (0)    | 0 (0)   | 0 (0)   |

345 L- larva; N- nymph; A- adult; M – male; F - female

346 *Bbsl* – *Borrelia burgdorferi* s.l.

347 LU = *B. lusitaniae*; VA = *B. valaisiana*; GA = *B. garinii*; AF = *B. afzelii*.

348 *B. burgdorferi* s.l infection was assessed through a nested PCR targeting the 5S-23S

349 rDNA intergenic spacer region (Rijpkema et al., 1995) and sequence analysis, followed

350 by a Reverse Line Blot (Gil 2005) on the positive samples, using specific probes

351 described previously (Ripkema 1995, Gil 2005, Gern 2010) to detect mixed infections.

352 For further details on methods see Supplemental Material 1.

353 **Table 2.** *Ixodes ricinus* ticks, skin biopsies and *B. lusitaniae* prevalence in ticks from lizards captured at Tapada de Mafra between March and  
 354 June 2013.

| Species                     | No. of individuals | No. of recaptures | No. of skin biopsies collected <sup>a</sup> | No. of infested | <i>Ixodes ricinus</i> collected from lizards |                                   |           |                                   |
|-----------------------------|--------------------|-------------------|---|-----------------|--|-----------------------------------|-----------|-----------------------------------|
|                             |                    |                   |   |                 | Larvae                                       |                                   | Nymphs    |                                   |
|                             |                    |                   |   |                 | No.  | <i>B. lusitaniae</i> prevalence % | No.       | <i>B. lusitaniae</i> prevalence % |
|                             |                    |                   |   |                 | collected                                    | (No. positives/ No. tested)       | collected | (No. positives/ No. tested)       |
| <i>Lacerta shreiberi</i>    | 1                  | 1                 | 1   | 1               | 1  | 0 (0/1)                           | 16        | 43.8 (7/16)                       |
| <i>Podarcis hispanica</i>   | 27                 | 1                 | 27  | 25              | 100  | 0 (0/99)                          | 12        | 16.7 (2/12)                       |
| <i>Psammodromus algirus</i> | 18                 | 0                 | 17  | 17              | 278  | 16.6 (33/199)                     | 19        | 21.0 (4/19)                       |
| <i>Timon lepidus</i>        | 2                  | 0                 | 2   | 0               | 0  | -                                 | 0         | -                                 |

355 <sup>a</sup> Biopsies were collected in duplicate except in 4 cases (2 collected only for direct DNA extraction, 2 only for culture in BSK II medium); Refer

356 to text for results on *Borrelia* prevalence in biopsies.

357 *B. burgdorferi* s.l prevalence was assessed through a nested PCR targeting the 5S-23S rDNA intergenic spacer region (Rijpkema et al., 1995)

358 and sequence analysis, followed by a Reverse Line Blot (Gil 2005) on the positive samples, using specific probes described previously (Ripkema

359 1995, Gil 2005, Gern 2010) to detect mixed infections. For further details on methods see Supplemental Material 1.

360



361 **Table 3.** Ixodidae ticks and skin biopsies collected from mammals at Tapada de Mafra sampled between March and June 2013.

| Species                      | No. of individuals | No. of recaptures | No. of skin biopsies <sup>a</sup> | No. of infested | Ticks collected from mammals <sup>b</sup> |                   |               |                      |        |
|------------------------------|--------------------|-------------------|-----------------------------------|-----------------|---|-------------------|---------------|----------------------|--------|
|                              |                    |                   |                                   |                 | Larvae                                    | <i>I. ricinus</i> |               | <i>I. acuminatus</i> |        |
|                              |                    |                   |                                   |                 | Larvae                                    | Nymphs            | Adult females | Larvae               | Nymphs |
| <i>Apodemus sylvaticus</i>   | 38                 | 26                | 36                                | 36              | 226                                       | 0                 | 0             | 3                    | 2      |
| <i>Crossidura russula</i>    | 1                  | 0                 | 1                                 | 1               | 3   | 0                 | 0             | 0                    | 0      |
| <i>Microtus lusitanicus</i>  | 2                  | 3                 | 2                                 | 2               | 2   | 0                 | 0             | 0                    | 0      |
| <i>Mus domesticus</i>        | 5                  | 1                 | 5                                 | 0               | 0   | 0                 | 0             | 0                    | 0      |
| <i>Mus spretus</i>           | 2                  | 0                 | 2                                 | 2               | 3   | 0                 | 0             | 0                    | 0      |
| <i>Oryctolagus cuniculus</i> | 1                  | 0                 | 1                                 | 1               | 1   | 0                 | 0             | 0                    | 0      |
| <i>Sus scrofa</i>            | 1                  | 0                 | 0                                 | 1               | 0   | 9                 | 2             | 0                    | 0      |

362 <sup>a</sup> Biopsies were collected in duplicate except in 4 cases (3 collected only for direct DNA extraction, 1 only for culture in BSK II medium); Refer

363 to text for results on *Borrelia* prevalence in biopsies.

364 <sup>b</sup> Refer to text for results on *Borrelia* prevalence in ticks.

365 **Table 4.** *Borrelia burgdorferi* s.l. infection prevalence in *Ixodes* sp. ticks collected  
 366 feeding on five *Turdus merula* captured from March to June 2013 at Tapada de Mafra.

| Tick family/<br>species | Stage | No. infected/<br>no. tested (%) | No. of ticks infected with <i>Bbsl</i> genospecies (%) |          |         |            |
|-------------------------|-------|---------------------------------|--|----------|---------|------------|
|                         |       |                                 | GA   | VA       | TU      | GA, VA, TU |
| Ixodidae                | N     | 1/1 (100)                       | 0 (0)  | 1 (100)  | 0 (0)   | 0 (0)      |
| <i>Ixodes ricinus</i>   | L     | 22/67 (32.8)                    | 13 (19.4)  | 8 (11.9) | 0 (0)   | 1 (1.5)    |
|                         | N     | 2/27 (7.4)                      | 1 (3.7)  | 0 (0)    | 1 (3.7) | 0 (0)      |
| <i>Ixodes frontalis</i> | L     | 1/1 (100)                       | 1 (100)  | 0 (0)    | 0 (0)   | 0 (0)      |
|                         | N     | 1/1 (100)                       | 0 (0)  | 0 (0)    | 1 (3.7) | 0 (0)      |

367 Ticks classified as Ixodidae lacked body structures needed for identification.

368 L- larva; N- nymph.

369 *Bbsl* – *Borrelia burgdorferi* s.l.

370 GA = *B. garinii*; VA = *B. valaisiana*; TU = *B. turdi*.

371 *B. burgdorferi* s.l prevalence was assessed through a nested PCR targeting the 5S-23S

372 rDNA intergenic spacer region (Rijpkema et al., 1995) and sequence analysis, followed

373 by a Reverse Line Blot (Gil 2005) on the positive samples, using specific probes

374 described previously (Ripkema 1995, Gil 2005, Gern 2010) to detect mixed infections.

375 For further details on methods see Supplemental Material 1.