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***Brucella papii* sp. nov. isolated from baboons (*Papio* spp.).**

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Running title: *Brucella papii* sp. nov. from baboons.

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1 **Abstract**

2

3 Two Gram-negative, non-motile, non-spore-forming coccoid bacteria (strains F8/08-60^T and
4 F8/08-61) isolated from clinical specimens obtained from baboons (*Papio* spp.) during that
5 had delivered stillborn offspring were subjected to a polyphasic taxonomic study. On the
6 basis of 16S rRNA sequence similarities both strains, which possessed identical sequences,
7 were assigned to the genus *Brucella*. This placement was confirmed by extended multilocus
8 sequence analysis (MLSA), where both strains possessed identical sequences, and whole
9 genome sequencing of a representative isolate. All the above analyses suggested that the
10 two strains represent a novel lineage within the genus *Brucella*. The strains also possessed
11 a unique profile when subjected to the phenotyping approach classically used to separate
12 *Brucella* species reacting only with *Brucella* A monospecific antiserum, being sensitive to the
13 dyes thionin and fuchsin, being lysed by bacteriophage Wb, Bk₂, and Fi phage at routine test
14 dilution (RTD) but only partially sensitive to bacteriophage Tb and with no requirement for
15 carbon dioxide, no production of hydrogen sulphide, but strong urease activity. Biochemical
16 profiling revealed a pattern of enzyme activity and metabolic capabilities distinct from
17 existing *Brucella* species. Molecular analysis of the *omp2* locus genes showed that both
18 strains had a novel combination of two highly identical *omp2b* gene copies. Both of the
19 strains shared a unique multiple copy IS711 fingerprint profile of this *Brucella* specific
20 element. Like MLSA, a multilocus variable number of tandem repeat analysis (MLVA)
21 showed that the isolates clustered very closely together, but represent a separate cluster
22 within the genus *Brucella*. Isolates F8/08-60^T and F8/08-61 could clearly be distinguished
23 from all known *Brucella* species and their biovars by both phenotypic and molecular
24 properties. Therefore, by applying the *Brucella* species concept suggested by the ICSP
25 Subcommittee on the Taxonomy of *Brucella*, they represent a novel species within the genus
26 *Brucella* for which the name *Brucella papii* sp. nov., with the type strain F8/08-60^T (= NCTC
27 XXXX^T = BCCN XXXX^T), is proposed.

28 **Body Text**

29 The genus *Brucella* currently comprises ten species *Brucella melitensis*, *Brucella abortus*,
30 *Brucella suis*, *Brucella canis*, *Brucella neotomae*, *Brucella ovis*, *Brucella pinnipedialis*,
31 *Brucella ceti*, *Brucella microti* and *Brucella inopinata* (Whatmore, 2009). The latter four
32 species have been described in the last decade after a long period of stability and reflect an
33 ongoing widening of the understanding of the host range of *Brucella* (Godfroid *et al*, 2011).
34 Due to high DNA-DNA relatedness of the six longstanding species (relatedness >70%) it had
35 been suggested that the genus should be monospecific and consist of only *B. melitensis* with
36 six biovars Melitensis, Abortus, Suis, Ovis, Neotomae, and Canis (Verger *et al.*, 1985).
37 However, in 2003, the ICSP Subcommittee on the Taxonomy of *Brucella* unanimously
38 agreed on a return to the pre-1986 *Brucella* taxonomy of six species with recognised biovars
39 of *B. suis*, *B. abortus* and *B. melitensis* (Osterman & Moriyon, 2006). Three of the newly
40 described species, *B. ceti*, *B. pinnipedialis* and *B. microti* (Foster *et al.*, 2007; Scholz *et al.*,
41 2008) conform to the existing pattern of high genetic homogeneity, for example all sharing
42 identical 16S rRNA sequences. However *B. inopinata*, although clearly much more closely
43 related to *Brucella* than to the nearest neighbour genus *Ochrobactrum* (De *et al*, 2008),
44 substantially extends the described genetic diversity within the group (Scholz *et al.*, 2010;
45 Wattam *et al.*, 2012; Wattam *et al.*, 2014). This has led some authors to informally divide the
46 genus into 'atypical' *Brucella*, including *B. inopinata* and other groups recently isolated from
47 rodents, amphibians and humans but yet to be formally taxonomically described (Tiller *et al.*,
48 2010a, Tiller *et al.*, 2010b; Eisenberg *et al.*, 2012; Fischer *et al.*, 2012), and 'core' *Brucella*,
49 representing the remaining nine genetically conserved species that cluster with the
50 longstanding 'classical' *Brucella* species.

51 Historically classification of *Brucella* species has been based on a combination of host
52 species preference and phenotypic biotyping that examines a range of characteristics
53 including CO₂ requirement, H₂S production, dye-sensitivity, lysis by *Brucella* specific
54 bacteriophage and agglutination with monospecific sera. Phenotyping is still used widely, at

55 least in reference laboratories, although its limitations are increasingly widely recognised and
56 the emergence of new species is further eroding its value. Recent descriptions have relied
57 heavily on additional data from emerging molecular approaches and a process of updating
58 the minimal standards to describe novel *Brucella* species, written almost 40 years ago
59 (Corbel and Brinley-Morgan, 1975), to reflect these approaches is ongoing within the current
60 ICSP Subcommittee on the Taxonomy of *Brucella* (www.the-icsp.org/subcoms/Brucella).

61 Here we describe the classification of two strains (AHVLA F8/08-60^T = Baboon 15719 =
62 NVSL 07-0026-1 and AHVLA F8/08-61 = Baboon 15331 = NVSL 07-0224-1) which were
63 originally isolated in 2006 and 2007 from two cases of stillbirth and retained placenta in
64 baboons at a primate research centre in Texas, USA (Schlabritz-Loutsevitch *et al.*, 2009).
65 The index case was a 13 year old baboon originally captured in Tanzania with a history of
66 three previous pregnancies including one abortion/stillbirth. The suspect *Brucella* (isolate
67 AHVLA F8/08-60^T) was isolated from a cervical swab obtained following a stillbirth in August
68 2006. Banked sera from the animal was serologically positive for *Brucella* using testing
69 validated for *B. abortus* in cattle (Brewers' Diagnostic Kits, Hynson, Westcott and Dunning
70 Inc, Baltimore, MD, USA). A second isolate was obtained from a swab of uterine content in
71 January 2007 one month after a stillbirth in an 8 year old colony born baboon with no
72 previous contact with the index case. This animal was serologically negative for *Brucella*.

73 Phenotype was examined by characterising growth on different media, CO₂ requirement,
74 H₂S production, growth in presence of dyes (thionin and basic fuchsin), reaction with
75 *Brucella* unabsorbed and monospecific A and M antisera and microscopy. Metabolic activity
76 in comparison with other *Brucella* was assessed using API20E, API20NE & APIZYM
77 (BioMerieux, France) with some confirmatory standalone biochemical testing and application
78 of the Micronaut[®] BfR *Brucella* assay (Al Dahouk *et al.*, 2010). Molecular analysis comprised
79 multiplex PCR, DNA-DNA hybridisation studies (Ziemke *et al.*, 1988), 16S rRNA sequence
80 analysis, *omp2a* and *omp2b* sequencing, multilocus sequence analysis (MLSA), multilocus
81 variable number of tandem repeat analysis (MLVA with 16 loci), and IS711 fingerprinting, all

82 of which have been used in recent descriptions of new *Brucella* species, with recently
83 completed whole genome sequencing supporting findings.

84

85 **Phenotypic Analysis**

86

87 Both isolates grow slowly on sheep blood agar (SBA) after incubation both aerobically and in
88 CO₂ with small, raised, circular, convex, non-haemolytic, greyish colonies, ~0.5mm in
89 diameter appearing after 3 days incubation at 37°C increasing to ~1mm in diameter after 6
90 days. Growth is poor at 28°C. On Farrell's agar growth also occurs without additional CO₂
91 with both isolates producing pinpoint colonies after 3 days incubation at 37°C becoming
92 cream in colour, circular, entire, convex and ~ 0.5-1mm in diameter after 6 days incubation.
93 On Serum Dextrose agar (SDA) growth is apparent after 24 hours at 37°C without additional
94 CO₂ with colonies of around 1 mm increasing to 3-4 mm after 72 hours incubation. Colonies
95 are smooth, entire and circular and show characteristic blue iridescence using obliquely
96 transmitted light (Henry, 1933). Growth is poor at 28°C. On nutrient agar there is poor growth
97 at 37°C and no growth at 28°C. Both isolates are sensitive to doxycycline (MIC <0.016
98 µg/ml), rifampicin (MIC 0.032 µg/ml), ciprofloxacin (MIC 0.094 µg/ml F8/08-60; 0.064 µg/ml
99 F8/08-61) and streptomycin (MIC 0.125 µg/ml F8/08-60; 0.094 µg/ml F8/08-61) in SDA and
100 fail to grow in nutrient both with 6% NaCl.

101

102 Both isolates are Gram-negative cocci, coccobacilli or short rods approximately 0.5-0.7µm in
103 diameter and 0.5-1.5 µm in length arranged singly or, occasionally, in pairs, small groups
104 and chains. Both isolates are consistent with *Brucella* using the modified Ziehl-Neelsen stain
105 (Brinley-Morgan *et al.*, 1978) being resistant to decolourisation with 0.5% acetic acid.
106 Flagellation was determined by transmission electron microscopy. Both isolates were dried
107 onto formvar/carbon coated support grids, which had been subjected to plasma glow
108 discharge, negatively stained with 2% phosphotungstic acid (pH6.6) and immediately

109 examined in a Phillips CM10 transmission electron microscope at 80 kV. Cells are
110 unflagellated coccobacilli approximately 0.5-1.0µm in length arranged as individual cells or
111 irregular clusters (Figure 1). Both isolates were examined by slide agglutination test using
112 unabsorbed *Brucella* antiserum (Weybridge Working Standard - WWS), negative control
113 serum and sterile normal saline and agglutinate with WWS8 but not with either the negative
114 control or saline.

115

116 Using classical phenotyping approaches that are traditionally used to divide *Brucella* into
117 species and biovars (Alton *et al.*, 1988) the organism does not conform to the characteristics
118 of any recognized species of *Brucella* (Table 1). The unusual profile includes no requirement
119 for carbon dioxide, no production of hydrogen sulphide, sensitivity to the dyes thionin and
120 basic fuchsin at 1:50,000, agglutination only with mono-specific anti-A serum, and lysis with
121 bacteriophage Wb, Bk₂ and Fi.

122

123 Both isolates were run through API20E, API20NE incubated aerobically at 37°C and 30°C
124 respectively and APIZYM (BioMerieux, France). As observed for other classical *Brucella*
125 species, and in contrast to the recently described *B. inopinata* and *B. microti*, the isolates
126 show limited metabolic reactivity. After 24 hrs incubation at 37°C API 20E strips for both
127 strains show positive reactions only for urea, VP, and fermentation of D-glucose and L-
128 arabinose. Both strips were re-incubated for a further 24 hours (not normally done under
129 manufacturer instructions) and this did produce a slight colour change in the sorbitol cupule
130 perhaps indicating slow fermentation in isolate F8/08-61. After 24 hrs incubation at 30°C the
131 API 20NE strips for both isolates were read - reactions only occurred in urea, there was no
132 reduction of nitrates or indole production. Reincubation of the API 20NE strips for a further
133 24 hours as per manufacturer instructions did not produce any changes to the profile of
134 either isolate. APIZYM strips were inoculated and incubated for 4.5 hours as per
135 manufacturer instructions. Both isolates produced identical results: alkaline phosphatase –;
136 esterase (C 4) ++; esterase lipase (C 8) +; lipase (C 14) –; leucine arylamidase ++; valine

137 arylamidase +; cystine arylamidase +; trypsin ++; α -chymotrypsin –; acid phosphatase ++;
138 naphthol-AS-bi-phosphohydrolase ++; α -galactosidase –; β -galactosidase –; β -glucuronidase
139 –; α -glucosidase –; B-glucosidase –; N-acetyl- β -glucosaminidase –; α -mannosidase – and α -
140 fucosidase – where + = positive (produced) ++ = positive (produced strong reaction) and – =
141 negative (not produced).

142

143 Oxidase and catalase production were examined. Both isolates produce catalase but are
144 negative for oxidase production by both pyotest strips (Medical Wire and Equipment, Bath,
145 UK) and freshly made oxidase reagent. Urea slopes were also inoculated and both isolates
146 rapidly hydrolyse urea within 30 minutes consistent with the positive urea results recorded
147 on API20E and API20NE. Nitrate broths were inoculated with both isolates and incubated for
148 24 hrs and 3 days at 37°C along with a positive control (*Serratia marcescens*), negative
149 control (*Acinetobacter lwoffii*) and an uninoculated broth. Both isolates do not reduce nitrate.

150

151 In addition to classical biochemical reactions extended metabolic activity was examined
152 using the Micronaut[®] BfR *Brucella* assay (Merlin Diagnostika, Bornheim-Hersel, Germany)
153 which assesses reaction with 93 different metabolites (Al Dahouk *et al.*, 2010). Once again,
154 and in contrast to *B. inopinata* and *B. microti* the isolates display limited metabolic reactivity,
155 especially in sugar metabolism, consistent with the ‘classical’ *Brucella* species group. The
156 metabolic profile is distinct from any extant *Brucella* species (Table 2) and hierarchical
157 cluster analysis (BioNumerics version 6.6, Applied Maths, Belgium), performed using the
158 Ward’s linkage algorithm (simple matching), confirmed this with the closest metabolic
159 relationship appearing to be with *B. suis* bv 5 (Figure 2). Within the same cluster but in a
160 different branch *B. canis*, *B. ovis*, *B. neotomae*, *B. ceti* and *B. pinnipedialis* are found.
161 Interestingly, the enzymatic reaction using H-hydroxyproline- β NA (hitherto defining the
162 genus *Brucella* together with a positive urease as well as negative Glu(pNA)-OH and Pyr-
163 pNA (Al Dahouk *et al.*, 2010; Al Dahouk *et al.*, 2012) for the first time revealed a negative
164 result. Hence metabolically *B. papii* sp. nov. differs from existing *Brucella* species.

165 **Molecular analysis**

166

167 The virtually complete 16S rRNA sequence was determined from both strains using an
168 approach described previously (Hunt *et al.*, 2013). Sequences of 1407bp obtained from both
169 isolates are identical to each other and confirm the identity of isolates as members of the
170 genus *Brucella*. Sequences show 2 bp differences from the nine 'core' *Brucella* species
171 previously shown to share identical 16S rRNA sequences (Gee *et al.*, 2004; Al Dahouk *et*
172 *al.*, 2012) and 7bp differences from *Brucella inopinata*, the only *Brucella* species previously
173 shown to have a divergent 16S rRNA sequence (Scholz *et al.*, 2010).

174

175 Results from DNA–DNA hybridizations using labelled DNA of *B. melitensis* 16M^T showed
176 79.1% ± 0.7 DNA relatedness with strain F8/08-60^T. These results are in agreement with
177 previous reports (Verger *et al.*, 1985) which showed that, if the 70% DNA-relatedness
178 threshold value is applied, species of this genus cannot be separated based on results from
179 DNA–DNA hybridizations. The DNA relatedness to the phylogenetically closest neighbour
180 *Ochrobactrum.intermedium* LMG 3301^T was 45.7%.

181

182 Both strains produce a strong band of identical size to the *B. melitensis* control in PCR
183 performed to determine the presence of the *Brucella* genus specific *bscp31* gene (Bailey *et*
184 *al.*, 1992). Fingerprinting using probes against the *Brucella* specific IS711 (Halling *et al.*,
185 1993) has been shown to be a useful tool for differentiating *Brucella* at species and/or sub-
186 species level (Ouahrani *et al.*, 1993; Bricker *et al.*, 2000; Dawson *et al.*, 2008). By Southern
187 blot the two baboon isolates share an identical high copy number IS711 profile that is distinct
188 from any profile seen previously (data not shown). Some of these IS711 copies have been
189 located in the genome sequence of one of the baboon isolates (Audic *et al.*, 2011). While
190 some IS711 chromosomal locations are common to other *Brucella* species but at least 7
191 may be specific to the baboon isolates.

192

193 Two frequently applied multiplex PCRs were applied to the isolates. AMOS (*B. abortus*, *B.*
194 *melitensis*, *B. ovis*, and *B. suis*) PCR (Bricker and Halling, 1994; Ewalt and Bricker, 2000;
195 Bricker *et al.*, 2003) with additional species-specific primers confirms that the isolates are
196 *Brucella* species because of the generation of the specific 180 bp amplicon. However, both
197 isolates fail to produce any of the expected PCR products that would identify them as a
198 known species (Schlabritz-Loutsevitch *et al.*, 2010). The strains were examined by
199 Bruceladder PCR, a multiplex PCR that can differentiate all known *Brucella* species (López-
200 Goñi *et al.*, 2008). The profile of bands obtained is identical to the *B. melitensis* profile (data
201 not shown) and thus the existing Bruceladder protocol would need to be adapted to
202 distinguish *B. papii* sp. nov. The novel IS711 localities described above give a means to do
203 this.

204

205 Studies of DNA polymorphism at the *omp2* locus have also been used extensively to
206 characterise *Brucella*. The baboon strains were shown uniquely to possess two almost
207 identical *omp2b* copies, as previously observed for *B. ceti*, but distinct from these latter by
208 being more *omp2b*-like at the 3' end of both *omp2* gene copies (Figure 3).

209

210 Both isolates were examined using an MLSA scheme that characterises the sequences of
211 21 independent genomic fragments equating to >10.2 Kbp (Al Dahouk *et al.*, 2012). Both
212 baboon isolates share identical sequences at all loci but possess unique alleles at 11/21 loci
213 not seen in characterisation of over 700 *Brucella* isolates representing all known species and
214 biovars. Phylogenetic analysis comparing the baboon isolates with type strains representing
215 all extant *Brucella* species and their biovars confirms that they represent a well separated
216 lineage most closely related to *B. ovis* (Figure 4).

217

218 Both isolates were typed using the MLVA assay described by Le Flèche *et al.* (2006) and Al
219 Dahouk *et al.* (2007) comprising 16 loci (Scholz & Vergnaud, 2013) with allele coding
220 convention following version 3.6 of the table for allele assignment at <http://mlva.u->

221 psud.fr/brucella/spip.php?rubrique29. All loci could be amplified with the exception of
222 Bruce11. The MLVA genotype is 2-5-0-14-2-2-5-3 for the 8 panel 1 loci (0 reflects lack of
223 amplification at locus Bruce11), 6-36-9 for panel 2A and 2-2-3-4-10 or 2-2-3-5-11 for the
224 most variable panel 2B. The two strains differ only at loci Bruce16 and Bruce30 typical of the
225 minor variation seen in closely related strains (Garcia-Yoldi *et al.*, 2006; Maquart *et al.*
226 2009). Figure 5 shows the relative position of the two strains compared to approximately
227 1900 different MLVA16 genotypes. In this minimum spanning tree (MST) connecting lines
228 longer than 6 are masked. Seven unconnected groups are identified. The largest one
229 contains *B. melitensis*, *B. abortus*, *B. pinnipedialis*, *B. ceti*, *B. suis* biovar 5, *B. microti*. The
230 second largest is made by *B. suis* biovar 2. The third group aggregates *B. canis*, and *B. suis*
231 biovars 1, 3, 4. *B. inopinata*, *B. ovis*, *B. neotomae* and *B. papii* (shown in red) constitute the
232 last four groups.

233

234 In addition one of the isolates (NVSL 07-0026-1) was subjected to whole genome
235 sequencing. Two independent comparative genomic analyses have been published based
236 on this sequence (Audic *et al.*, 2011; Wattam *et al.*, 2014) comparing this strain with all other
237 *Brucella* species type strains. Both confirm the phylogenetic position suggested by MLSA
238 showing the baboon isolate and *B. ovis* united by an extremely shallow branch indicating a
239 shared common ancestor but with the long branch length indicative of significant divergence
240 since that time (Figure 6). Further, the *in silico* MLVA genotyping tool accessible at
241 <http://mlva.u-psud.fr> was applied to the whole genome sequence (WGS). The deduced code
242 was exactly as determined by PCR and electrophoresis size measurement, illustrating that
243 *Brucella* tandem repeats have been correctly assembled from the initial 454 WGS sequence
244 data. Investigation of the WGS data shows that one of the two flanking sequences of the
245 Bruce11 VNTR contains an approximately 141 bp deletion (101 bp of flanking sequence,
246 40bp out of 63 bp of the first repeat unit, plus potentially an unknown number of full repeat
247 units). This explains the lack of amplification of Bruce11 in *B. papii* since one of the usual
248 Bruce11 PCR primers is within this deletion. If primer GTAGCGATTGACACCTTGCCTG is

249 used instead of CTGTTGATCTGACCTTGCAACC, an identical 396 bp PCR product is
250 obtained in both strains whereas the Bruce11 PCR product is 93 bp longer in the other
251 *Brucella* (data not shown).

252

253 **Conclusions**

254

255 In summary, extensive phenotypic and genotypic analyses demonstrate that isolates F8/08-
256 60^T and F8/08-61 are members of the genus *Brucella*, but can be clearly differentiated from
257 all established species of this genus, including their biovars. Hence, by applying the *Brucella*
258 species concept suggested by the ICSP Subcommittee on the Taxonomy of *Brucella*
259 (Osterman & Moriyon, 2006), the two strains represent a novel species of the genus
260 *Brucella*, for which we propose the name *Brucella papii* sp. nov.

261

262 **Description of *Brucella papii* sp. nov.**

263

264 *Brucella papii* (L. gen. n. papii, of the baboon, from which the first strains of this species
265 have been isolated).

266

267 Cocci, coccobacilli or short rods, ~0.5-0.7µm in diameter, ~0.5-1.5 µm in length, arranged
268 singly and, occasionally in pairs, small groups and chains. Gram negative and resistant to
269 decolourisation with 0.5% acetic acid. Non-motile and non-spore forming. Aerobic. Does not
270 require supplementary CO₂ for growth. Growth occurs at 30°C to 37°C. Colonies on sheep
271 blood agar and Farrell's are visible at 3-4 days and are small, raised, circular, entire and
272 convex of ~0.5-1mm in diameter. Non-haemolytic and greyish in colour (on blood agar) and
273 honey coloured (on Farrell's). No growth occurs on MacConkey agar. No growth in broth
274 with 6.5% NaCl. Isolates do not grow in the presence of thionin or basic fuchsin at 1/50 000.
275 Both strains agglutinate with monospecific anti-A serum but not anti-M or anti-R serum. Cells
276 are lysed by Wb, Bk₂, Tb and Fi phage at routine test dilution (RTD). Cells are sensitive to

277 doxycycline, rifampicin, ciprofloxacin and streptomycin. Catalase positive. Oxidase negative.
278 Strongly urease positive. Indole negative. No production of H₂S. Acetoin (Voges Proskauer)
279 is produced. Nitrates are not reduced. No production of arginine dihydrolase, lysine
280 decarboxylase, ornithine decarboxylase, β-galactosidase, β-glucosidase or gelatinase.
281 Positive (API20E) for L-arabinose, D-glucose (weak at 37°C but not 30°C) and variable, but
282 weak, fermentation of D-sorbitol. No fermentation or oxidation of D-mannitol, inositol, L-
283 rhamnose, D-sucrose, D-melibiose or amygdalin at 37°C. No assimilation of D-glucose, L-
284 arabinose, D-mannose, D-mannitol, D-maltose, N-acetyl-glucosamine, potassium gluconate,
285 capric acid, adipic acid, malic acid, trisodium citrate or phenylacetic acid at 30°C. Esterase
286 (C 4), esterase lipase (C 8), leucine arylamidase, valine arylamidase, cystine arylamidase,
287 trypsin, acid phosphatase, and naphthol-AS-Bi-phosphohydrolase are all produced. Alkaline
288 phosphatase, lipase (C 14), α-chymotrypsin, α-galactosidase, β-glucuronidase, α-
289 glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase and tryptophane
290 deaminase are not produced. Differentiating physiological reactions (Micronaut[®] BfR
291 *Brucella* assay) of strains F8/08-60^T and F8/08-61 with respect to other *Brucella* species are
292 given in Table 2.

293 The type strain is F8/08-60^T (BCCN XXXX^T NCTC XXXX^T) isolated in 2006 from a cervical
294 swab taken from a *Papio* spp. following stillbirth in an animal handling facility in Texas, USA.

295

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297

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312

313 **Nucleotide Accession Numbers**

314

315 The EMBL accession numbers for the gene sequences of 16S rRNA and *omp2a/omp2b* are
316 HG932316/HG932317 and KJ493822, KJ493823, KJ510540 and KJ510541 respectively.

317

318 **Figure and Table Legends**

319

320 Table 1. Differentiating characteristics of *B. papii* from other *Brucella* species and biovars
321 based on classical biotyping approaches.

322

323 Table 2. Metabolic activity of *Brucella papii* sp. nov. in comparison with classical and newly
324 described species tested with the Micronaut[®] BfR *Brucella* assay. (-, no metabolic activity;
325 +, metabolic activity; v, variable metabolic activity. *Brucella*-specific traits are shown in
326 boldface, and potentially distinctive features of *Brucella papii* sp. nov. are shaded).

327 Figure 1. Electron micrographs showing non-flagellated F8/08-61 cells. The appearance of
328 F8/08-60^T is identical.

329

330 Figure 2. Hierarchical cluster analysis of *Brucella* species including *Brucella papii* sp. nov.
331 performed by the Ward's linkage algorithm (simple matching) on the basis of 93 biochemical
332 reactions tested with the Micronaut[®] BfR *Brucella* assay.

333

334 Figure 3. Schematic multiple nucleotide sequence alignment of the *omp2a* and *omp2b*
335 genes of *Brucella* strains: Nucleotide sequences are represented by large rectangles divided
336 into boxes of 30 nucleotides. *B. microti omp2b* gene is taken as the reference sequence.
337 The yellow colour is typical of *B. microti omp2a*-specific nucleotides. The numbers in the
338 corresponding boxes indicate the number of *omp2a*-specific nucleotides present in the
339 sequence considered. The numbers in parentheses show insertions and deletions. The
340 numbers in red indicate nucleotide differences that are not due to gene conversion.

341

342 Figure 4. Phylogenetic analysis of the baboon isolates in comparison with type strains (see
343 <http://www.the-icsp.org/taxa/Brucellalist.htm> for details of strains) of all *Brucella* species and
344 biovars inferred by MLSA. Bar = 0.002 substitutions per nucleotide position. Analysis was
345 performed with the MEGA5 package using the neighbour-joining approach.

346

347 Figure 5. Minimum spanning tree from the MLVA16 data. Published MLVA16 data which can
348 be accessed from the cooperative *Brucella* MLVA database at <http://mlva.u-psud.fr> was
349 compared to the *B. papii* MLVA16 data. The colour code reflects species and sometimes
350 biovar or significant sub-species clustering. The two *B. papii* strains are shown in red.
351 Categorical distance was used. The creation of hypothetical intermediate links was allowed.
352 Only connecting branches up to a length of 6 are shown.

353

354 Figure 6. Phylogenetic representation of species in the genus *Brucella* based on a
355 concatenated alignment of orthologous genes of complete or nearly complete genome
356 sequences of 34 *Brucella* strains, including *B. papii* sp. nov.

357

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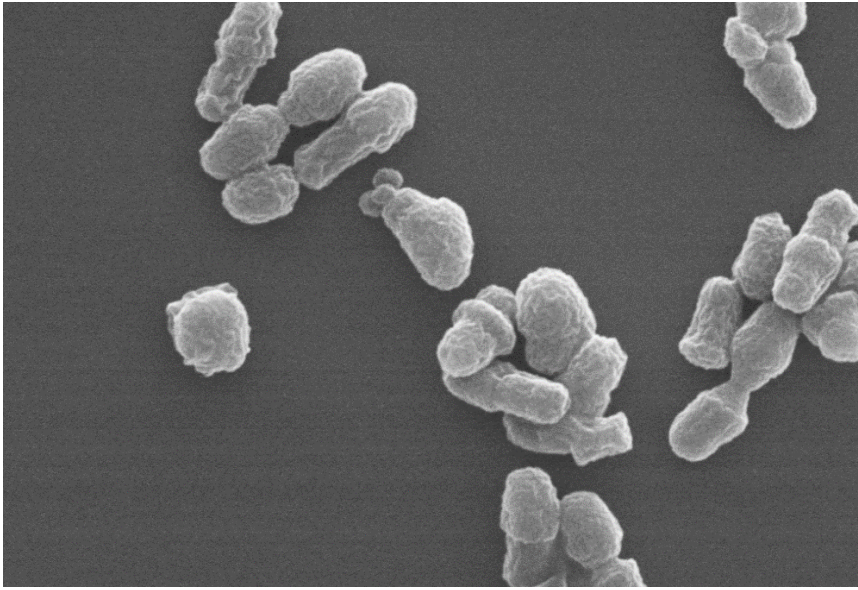
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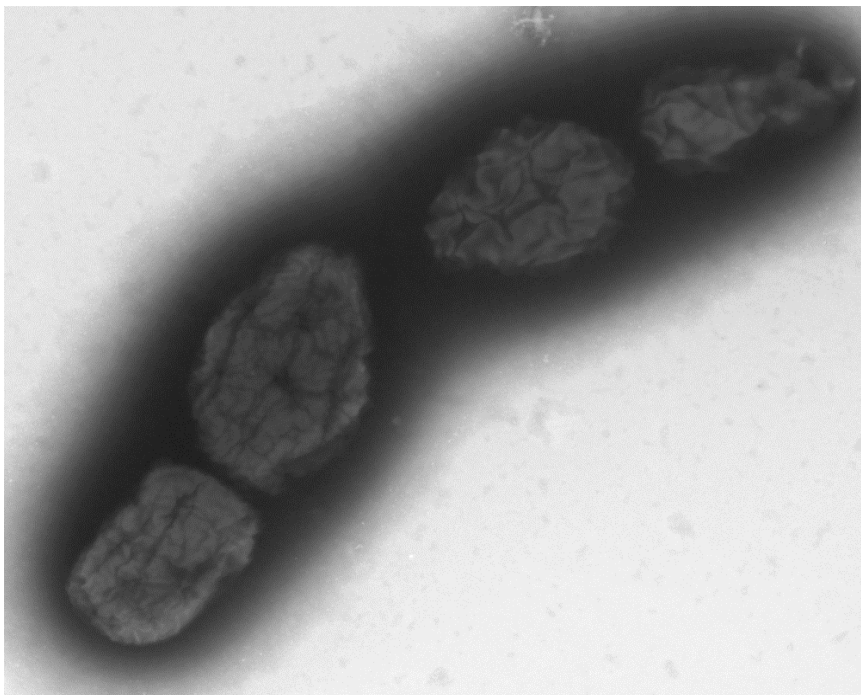
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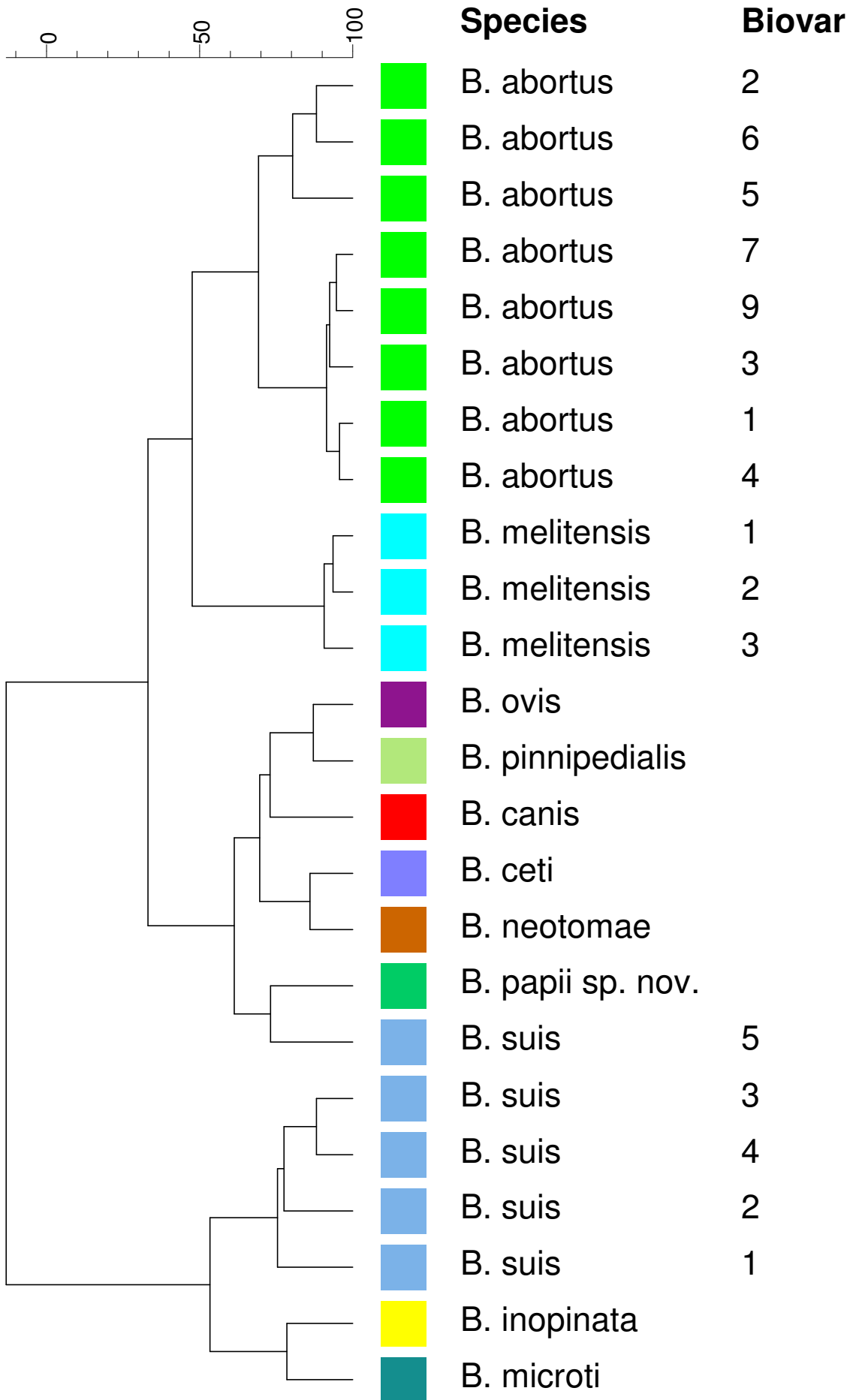
Figure 1



— 1 μ m

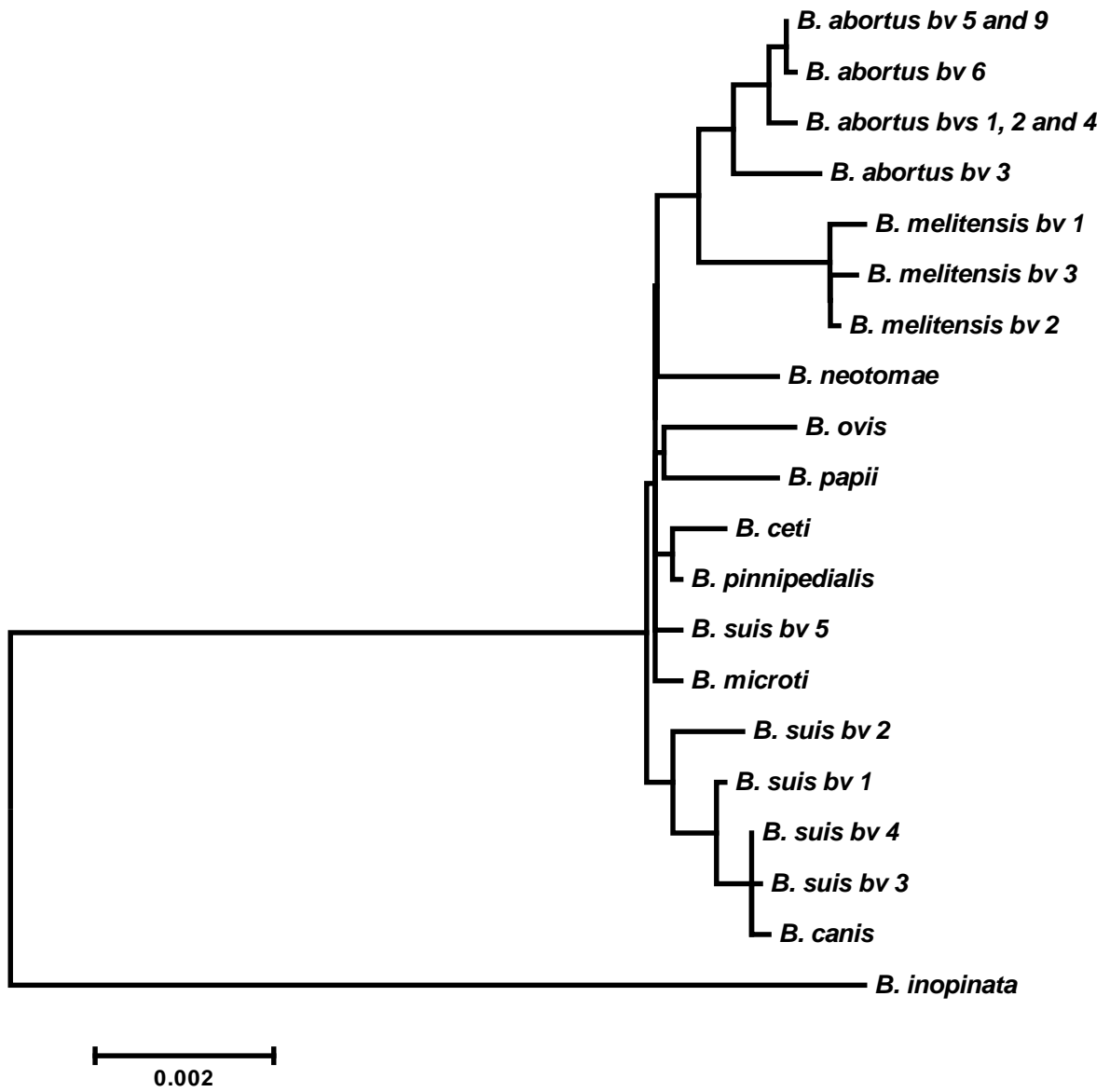


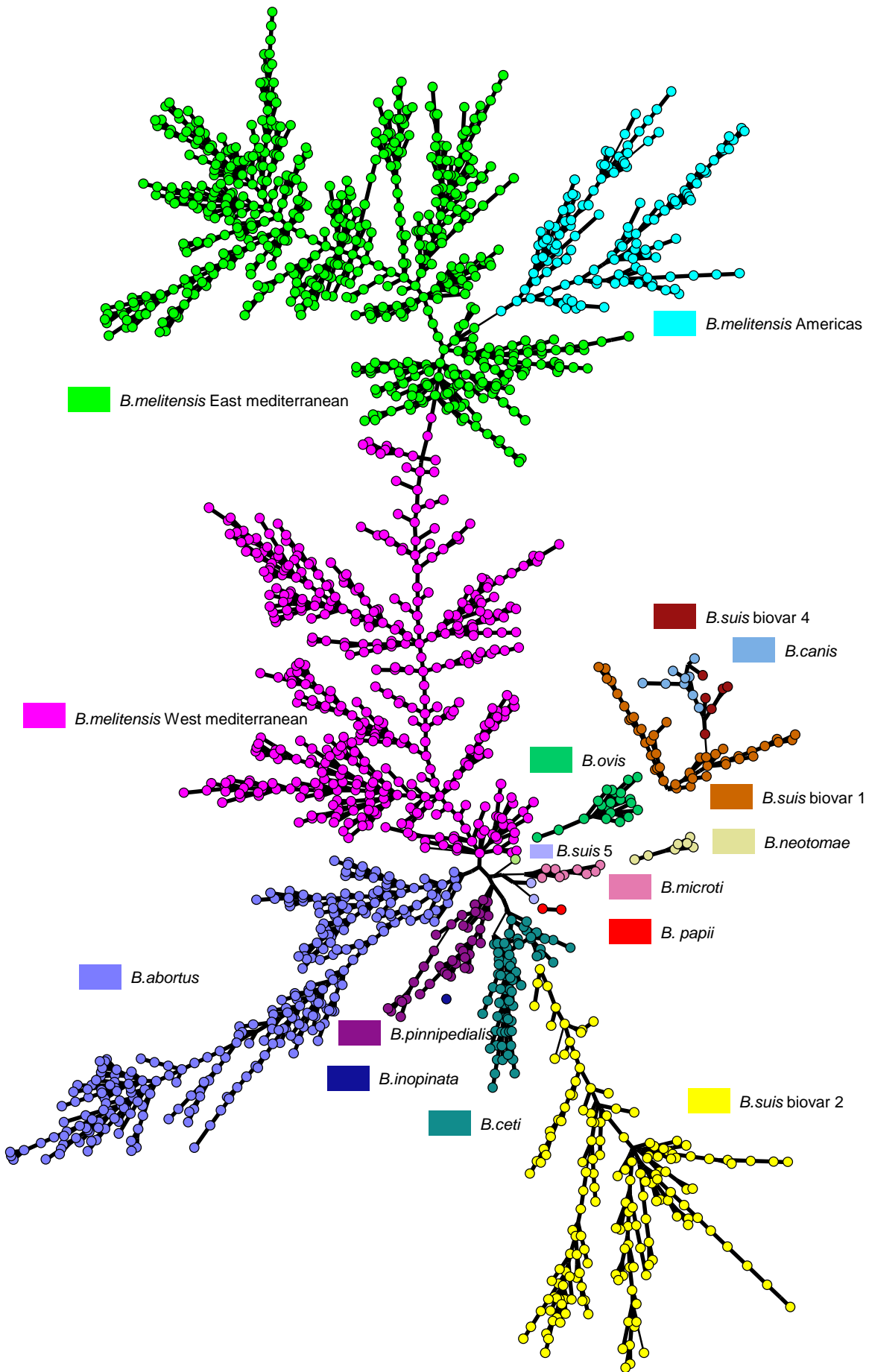
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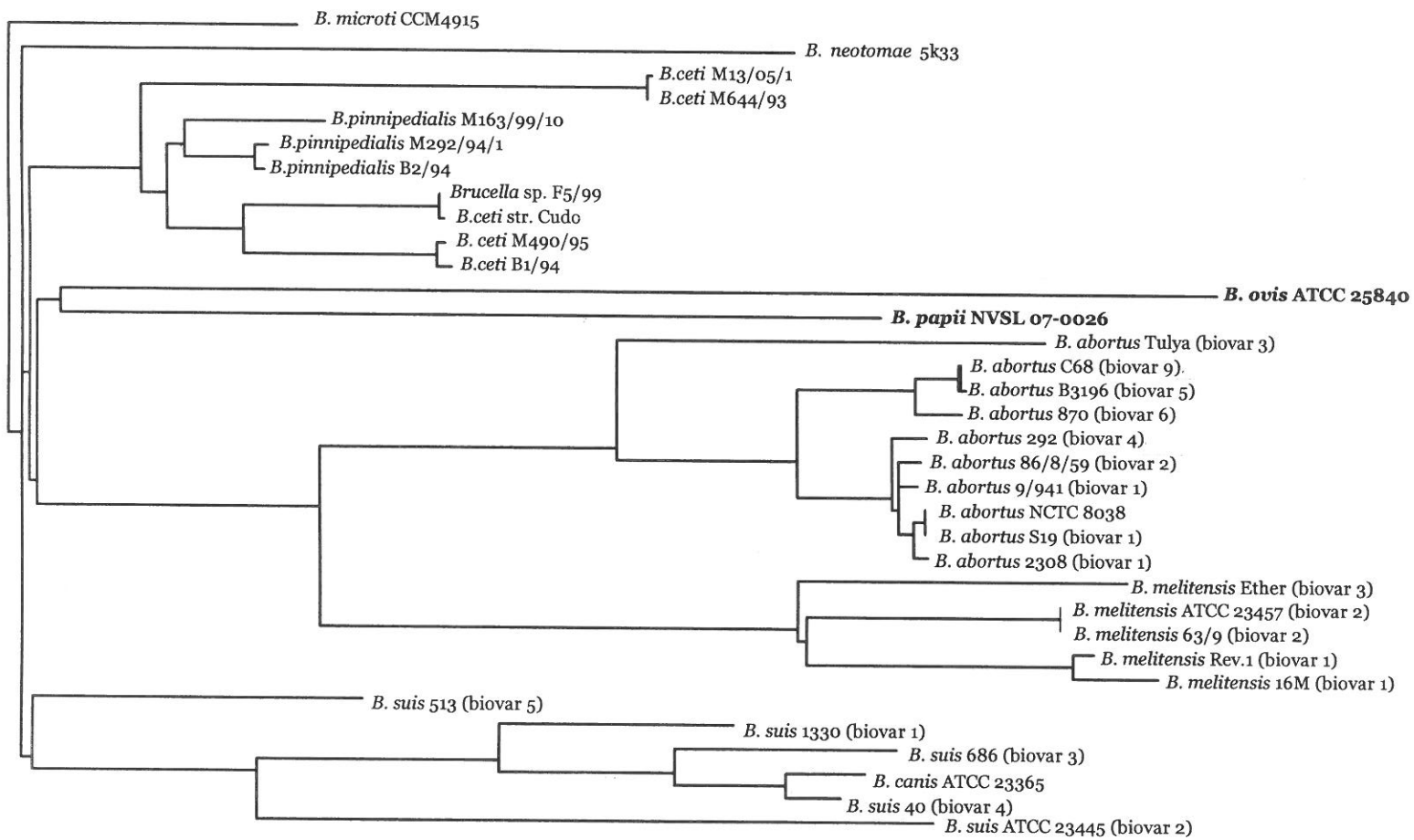


GenBank Accession N°	Species	Strain	Host or source	omp2gene		90	180	270	360	450	540	630	720	810	900	990	1080	1140																				
AM943813	<i>B. microti</i>	BMS 20	Soil	omp2b																																		
AM943807				omp2a	1		4	1	9	1	10	9	12	5 (12)	6 (18)	8	7	1	6	2	1	1	8	(8)	3	(7)	1	2	2	5	14	1	4	7	1	2	2	
U26440	<i>B. melitensis</i>	16M	Goat	omp2b	1																																	
U26440				omp2a	1																																	
U26443	<i>B. suis</i>	1330	Swine	omp2b	1																																	
U26443				omp2a	1																																	
AF268035	<i>B. ovis</i>	76-250	Sheep	omp2b	1		4	1	9	1	10	8	11	1	5 (12)	6 (18)	8	7	1	6	2	1	1	8	(8)	3	(7)	1	2	2	5	14	1	4	7	1	2	2
AY008720				omp2a	1		4	1	9	1	10	9	12	5 (12)	6 (18)	8	7	1	1	2	1	1	8	(8)	3	(7)	1	2	2	5	14	1	4	7	1	2	2	
U26442	<i>B. ovis</i>	63/290	Sheep	omp2b	1		4	1	9	1	10	8	12	5 (12)	6 (18)	8	7	1	1	2	1	1	8	(8)	3	(7)	1	2	2	5								
U26442				omp2a	1		4	1	9	1	10	9	12	5 (12)	6 (18)	8	7	1	1	2	1	1	8	(8)	3	(7)	1	2	2	5	14	1	4	7	1	*	*	
AF300816	<i>B. ceti</i>	B1/94	Porpoise	omp2b	1		4	1	9																													
AF300817				omp2a	1		4	1	9																													
AF300814	<i>B. ceti</i>	B14/94	Common dolphin	omp2b	1																																	
AF300815				omp2a	1																																	
KJ510540	<i>B. papii</i> sp. nov.	F8/08/60	Baboon	omp2b	1																														1	2	2	
KJ493822				omp2a	1																														1	2	2	
KJ510541	<i>B. papii</i> sp. nov.	F8/08/61	Baboon	omp2b	1																														1	2	2	
KJ493823				omp2a	1																														1	2	2	

Figure 4:







0.001

Species	Biotype	Urease	CO ₂ rq't	H ₂ S prod'n	Growth on media containing:		Agglutination with monospecific antisera			Lysis by phage at RTD ²						
					Thionin ¹	Fuchsin ¹	A	M	R	Tb	Wb	Bk ₂	Fi	R/C	Tb10 ⁴	
<i>B. papii</i> sp. nov.		+	-	-	-	-	+	-	-		PL or NL	L	L	L	NL	PL
<i>B. abortus</i>	1	(+)*	(+)	+	-	+	+	-	-		L	L	L	L	NL	L
	2	+	(+)	+	-	-	+	-	-		L	L	L	L	NL	L
	3**	+	(+)	+	+	+****	+	-	-		L	L	L	L	NL	L
	4	+	(+)	+	-	+	-	+	-		L	L	L	L	NL	L
	5	+	-	-	+	+	-	+	-		L	L	L	L	NL	L
	6**	+	-	(+)	+	+	+	-	-		L	L	L	L	NL	L
	7***	+	-	(+)	+	+	+	+	-		L	L	L	L	NL	L
	9	+	-	+	+	+	-	+	-		L	L	L	L	NL	L
	<i>B. suis</i>	1	+	-	+	+	-*****	+	-	-		NL	L	L	PL	NL
2		+	-	-	+	-	+	-	-		NL	L	L	PL	NL	L
3		+	-	-	+	+	+	-	-		NL	L	L	PL	NL	L
4		+	-	-	+	(-)	+	+	-		NL	L	L	PL or L	NL	L
5		+	-	-	+	-	-	+	-		NL	L	L	PL	NL	L
<i>B. melitensis</i>	1	+	-	-	+	+	-	+	-		NL	NL	L	NL	NL	NL
	2	+	-	-	+	+	+	-	-		NL	NL	L	NL	NL	NL
	3	+	-	-	+	+	+	+	-		NL	NL	L	NL	NL	NL
<i>B. ovis</i>		-	+	-	+	(+)	-	-	+		NL	NL	NL	NL	L	NL
<i>B. canis</i>		+	-	-	+	-	-	-	+		NL	NL	NL	NL	L	NL
<i>B. neotomae</i>		+	-	+	-	-	+	-	-		NL or PL	L	L	L	NL	L
<i>B. ceti</i>		+	(-)	-	(+)	(+)	+	(-)	-		NL ^α	L ^β	L ^β	NL or PL	NL	
<i>B. pinnipedialis</i>		+	(+)	-	+	+	(+)	(-)	-		NL ^α	L ^β	L ^β	NL or PL	NL	
<i>B. microti</i>		+	-	-	+	+	-	+			NL	L		NL		L
<i>B. inopinata</i>		+	-	+	+	+	-	+*****			NL			NL		PL

¹Concentration = 1/50 000 w/v.

²Phage lysis: RTD = Routine Test Dilution Tb = Tbilisi Wb = Weybridge Bk₂ = Berkeley Fi = Firenze R/C = Rough strains Tbx10⁴ = RTD x 10⁴.

L = Confluent Lysis, PL = Partial Lysis, NL = No Lysis, α Most strains no lysis, β Most strains lysis.

(+) = Most strains positive, (-) = most strains negative.

*Reference strain is -ve but most field strains are positive.

**For more certain differentiation of biotype 3 and 6, thionin at 1/25 000 (w/v) is used in addition. Biovar 3 = +, Biovar 6 = -.

***The status of biovar 7 is under investigation (Garin-Bastuji *et al.*, 2014).

****Some strains of this biotype are inhibited by basic fuchsin, *****Some isolates may be resistant to fuchsin, ***** Weak agglutination.

β -Ala- β NA	β A	aminopeptidases with β NA	-	-	-	-	-	-	-	-	-	-	-
H-Val-4-M β NA	V4M	aminopeptidases with β NA	-	-	-	-	-	-	-	-	-	-	-
bis-p-nitrophenyl phosphate	BISPH7	phosphatases	-	-	V	-	-	-	-	-	+	+	-
p-nitrophenyl phosphate di(2-amino-2-ethyl-1,3-propanediol)	PHOS7	phosphatases	-	-	-	-	-	-	-	-	+	+	-
p-nitrophenyl- α -D-glucopyranoside	aGLU7	glucosidases	-	-	+	-	V	+	-	-	+	+	-
p-nitrophenyl- α -D-maltoside	aMAL7	glucosidases	-	-	-	-	-	-	-	-	+	+	-
p-nitrophenyl- α -D-glucopyranoside	aGLU5	glucosidases	-	-	V	-	-	-	-	-	+	+	-
p-nitrophenyl- α -D-xylopyranoside	aXYL7	glucosidases	-	-	+	-	-	-	-	-	+	+	-
p-nitrophenyl-n-acetyl- β -D-glucosaminide	CHIT7	esterases	-	-	V	-	-	-	-	-	+	+	-
i-erythritol	EROL	sugar derivates	+	+	V	-	-	+	V	-	+	V	-
L(-)-fucose	L-FUC	monosaccharides	-	V	-	-	V	-	V	-	+	+	-
D-glucose-L-cysteine	GLUCY	sugar derivates	V	V	+	-	-	+	V	V	+	+	V
D(-)-ribose	D-RIB	monosaccharides	V	-	V	-	+	-	-	-	+	V	-
D(-)-arabinose	D-ARA	monosaccharides	-	V	-	-	-	-	-	-	V	V	-
D(+)-glucose	D-GLU	monosaccharides	-	V	V	-	-	+	-	-	+	V	-
L(+)-arabinose	L-ARA	monosaccharides	V	-	V	-	-	+	V	-	+	+	-
2-deoxy-D-galactose	DOGAL	monosaccharides	-	-	-	-	-	-	-	-	-	-	-
D(+)-galactose	D-GAL	monosaccharides	V	-	V	-	-	-	-	-	+	+	-
D(+)-xylose	D-XYL	monosaccharides	-	-	V	-	-	-	-	-	+	-	-
α -D-talose	D-TAL	monosaccharides	+	+	+	-	-	+	+	-	+	-	-
D-threitol	D-TOL	sugar derivates	+	-	V	V	+	-	+	-	+	+	-
adonite	ADON	monosaccharides	+	+	V	V	-	+	+	+	+	-	+
sucrose	SUCR	disaccharides	-	-	+	-	+	-	-	-	+	+	-
iso-maltose	MALTO	disaccharides	-	-	+	-	+	-	-	V	+	+	-
D(-)-fructose	D-FRU	monosaccharides	V	+	+	-	+	+	+	-	+	+	+
1-deoxy-1-nitro-D-sorbitol	DNSOL	sugar derivates	-	-	-	-	-	-	-	-	-	-	-
D(-)-lyxosylamine	LYXA	sugar derivates	-	-	+	-	+	+	+	-	+	+	+
palatinose	PAL	disaccharides	-	-	+	-	+	-	-	-	+	+	-
myo-inositol	INOL	sugar derivates	-	-	-	-	-	-	-	-	-	+	-
inosine	INON	sugar derivates	-	-	V	-	+	-	+	-	+	-	-
gallic acid	Galli	organic acids	-	+	+	-	+	+	V	-	+	V	+
DL-lactic acid	dILac	organic acids	-	-	+	-	-	-	-	-	+	V	-
acetate	Acet	amino acid derivates	-	-	+	+	+	+	-	-	+	+	+

L-asparagine	L-Asn	amino acids	+	+	V	-	-	-	-	-	+	+	-
L-glutamic acid	L-Glu	amino acids	V	+	V	-	V	+	-	-	+	+	-
ala-gln	AlaGln	amino acids	V	-	-	-	-	-	-	-	+	V	-
guanidinosuccinic acid	GuaSu	organic acids	V	-	V	-	V	-	-	-	+	V	-
L-cystine	L-Cyss	amino acids	+	+	V	+	-	-	V	+	+	-	V
D-alanine	D-Ala	amino acids	V	+	V	-	-	+	-	-	+	+	-
propionic acid	Propn	organic acids	-	+	V	-	-	+	V	-	-	+	-
L-alanine	L-Ala	amino acids	+	+	V	-	V	-	-	-	+	+	-
DL-β-hydroxybutyric acid	βHBut	organic acids	-	-	+	-	+	+	+	-	+	+	+
D-asparagine	D-Asn	amino acids	V	+	-	-	-	+	-	-	-	V	-
L-arginine	L-Arg	amino acids	-	-	+	-	V	-	-	-	+	+	-
Na-acetyl-l-arginine	AcArg	amino acid derivates	-	-	+	-	+	-	-	-	V	+	-
glyoxylic acid	Glyx	organic acids	-	-	-	-	-	-	-	-	-	+	-
L-serine	L-Ser	amino acids	-	V	-	-	-	-	-	-	+	+	-
hippuryl-arg	HipArg	peptides	-	-	V	-	V	-	-	-	V	+	-
L-carnosine	L-Caro	amino acids	-	-	V	-	-	-	-	-	+	+	-
glycine	Gly	amino acids	-	-	-	-	-	-	-	-	+	+	-
adipic acid	Adipa	organic acids	-	-	V	-	+	-	+	-	+	+	-
L-proline	L-Pro	amino acids	-	+	-	-	-	-	-	-	+	+	-
D-proline	D-Pro	amino acids	-	+	-	-	-	-	-	-	+	+	-
fumaric acid	Fuma	organic acids	-	-	-	-	-	-	-	-	+	+	-
D-serine	D-Ser	amino acids	-	+	-	-	-	-	-	-	-	-	-
glycolic acid	Glyc	organic acids	-	-	-	-	-	-	-	-	-	+	-
Adenine	Adeni	amino acid derivates	+	+	V	-	+	-	+	V	-	-	+
glutaric acid	Gluta	organic acids	-	-	-	-	-	-	-	-	+	+	V
mesaconic acid	Mesac	organic acids	-	-	-	-	-	-	-	-	+	-	-
D-histidine	D-His	amino acids	-	-	-	-	-	-	-	-	-	+	-
nitrite	NTI	classical reactions	-	-	-	-	-	-	-	-	+	+	-
nitrate	NTA	classical reactions	+	V	+	-	V	-	+	+	+	V	-
pyrazinamidase	PCA	classical reactions	V	+	V	-	-	+	+	+	+	V	+
Voges Proskauer	VP	classical reactions	+	+	V	-	+	+	+	+	+	+	+
Urease	Urease	classical reactions	+	+	+	+	+	+	+	+	+	+	+
hydrogen sulfide	H2S	classical reactions	-	-	V	-	-	-	-	-	+	+	-