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2 **Intercontinental diversity of *Caballeronia* gut symbionts in the conifer pest bug**

3 ***Leptoglossus occidentalis***

4

5 Tsubasa Ohbayashi^{1,2**}, Raynald Cossard^{2†}, Gaëlle Lextrait², Takahiro Hosokawa³,

6 Vincent Lesieur⁴, Kazutaka Takeshita⁵, Kanako Tago¹, Peter Mergaert², and Yoshitomo

7 Kikuchi^{6,7}

8 ¹Institute for Agro-Environmental Sciences, National Agriculture and Food Research

9 Organization (NARO), 305-8604, Tsukuba, Japan

10 ²Institute for Integrative Biology of the Cell, UMR9198, CEA, CNRS, Université Paris-

11 Saclay, 91198 Gif-sur-Yvette, France

12 ³Department of Biology, Faculty of Science, Kyushu University, 819-0395 Fukuoka,

13 Japan

14 ⁴CSIRO Health and Biosecurity, European Laboratory, Montferrier sur Lez, France

15 ⁵Faculty of Bioresource Sciences, Akita Prefectural University, 010-0195 Akita, Japan

16 ⁶Bioproduction Research Institute, National Institute of Advanced Industrial Science and

17 Technology (AIST), Hokkaido Center, Sapporo 062-8517, Japan

18 ⁷Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

19 [†]These authors contributed equally to this work

20 * Corresponding author:

21 Tsubasa Ohbayashi: obayashit472@affrc.go.jp; +81- 29-838-8309

22 **Abstract (<250 words)**

23 Many stinkbugs in the superfamily Coreoidea (Hemiptera: Heteroptera) develop crypts
24 in the posterior midgut, harboring *Caballeronia* (*Burkholderia*) symbionts. These
25 symbionts form a monophyletic group in *Burkholderia* sensu lato, called the “stinkbug-
26 associated beneficial and environmental (SBE)” group, recently reclassified as the new
27 genus *Caballeronia*. The SBE symbionts are separated into the subclades SBE- α and
28 SBE- β . Previous studies suggested a regional effect on the symbiont infection pattern:
29 Japanese and American bug species tend to be associated with the SBE- α , while
30 European bug species are almost exclusively associated with the SBE- β . However,
31 since only a few insect species have been investigated, it remains unclear whether the
32 region-specific infection trend is general or not. We herein investigated *Caballeronia*
33 gut symbionts in diverse Japanese, European, and North American populations of a
34 cosmopolitan species, the Western conifer seed bug *Leptoglossus occidentalis*
35 (Coreoidea: Coreidae). Molecular phylogenetic analysis of the 16S rRNA gene
36 demonstrated that the SBE- β was the most dominant in all populations. Notably, SBE- α
37 was rarely detected in any region, while a third clade, the “Coreoidea-clade” occupied
38 one fourth of the tested populations. While aposymbiotic bugs showed a high mortality,
39 both SBE- α - and SBE- β -inoculated insects showed high survival rates, but a
40 competition assay demonstrated that the SBE- β outcompeted the SBE- α in the midgut
41 crypts of *L. occidentalis*. These findings suggest that the symbiont specificity in the
42 *Leptoglossus-Caballeronia* symbiotic association is determined by the host rather than

43 geography while in other bugs, geographic distribution of symbionts might be more

44 important.

45

46 Key words: *Caballeronia*, stinkbug, obligate gut symbiosis, intercontinental diversity

47

48 Running headline: Diversity of *Caballeronia* bug symbionts

49

50

51 **Introduction**

52 Recent studies have revealed that insect gut microorganisms play a pivotal role
53 in evolution and environmental adaptation of insects. Gut microorganisms provide
54 essential nutrients, digest indigestible food materials, and/or degrade phytotoxins and
55 insecticides (Engel and Moran, 2013; Kikuchi *et al.*, 2012; Itoh *et al.* 2018; Moran *et*
56 *al.*, 2019; Salem *et al.*, 2014; Sudakaran *et al.*, 2017). Many species of stinkbugs in the
57 superfamily Coreoidea (Hemiptera: Heteroptera) develop numerous crypts at the
58 posterior part of the midgut, wherein specific *Caballeronia* symbionts (previously
59 included in the genus *Burkholderia*) are densely proliferating - usually as a single
60 species - until almost full occupation of the luminal space (Acevedo *et al.*, 2021; Garcia
61 *et al.*, 2014; Hunter *et al.*, 2022; Kikuchi *et al.*, 2005, 2011a; Kuechler *et al.*, 2016;
62 Ohbayashi *et al.*, 2019b; Olivier-Espejel *et al.*, 2011; Takeshita and Kikuchi 2017). The
63 *Caballeronia* gut symbionts play an important role for their hosts such as recycling
64 metabolic waste materials and providing essential amino acids and vitamins, thereby
65 enhancing growth and fecundity of the stinkbugs (Ohbayashi *et al.*, 2019a; Kikuchi *et*
66 *al.*, 2007; Kikuchi and Fukatsu, 2014). A particularity of this mono-species symbiosis is
67 the horizontal transmission of the symbionts. Indeed, hatchlings are symbiont-free and
68 acquire the *Caballeronia* symbionts from the soil during the early instar stages (Kikuchi
69 *et al.*, 2007; 2011b; Ohbayashi *et al.*, 2019b). This implies that the insects depend on
70 efficient and stringent selection mechanisms to sort environmental bacteria in order to
71 give access to *Caballeronia* symbionts only (Itoh *et al* 2019; Kikuchi *et al* 2020;

72 [Ohbayashi et al 2015](#)) but also that the geographic distribution of symbiont species
73 could be determinant for the outcome of the symbiosis ([Ohbayashi et al., 2019b](#)).

74 The genus *Burkholderia* was firstly separated from *Pseudomonas* Group II in
75 1991 ([Yabuuchi et al., 1991](#)), into a heterogeneous taxonomic group with more than
76 100 bacterial species ([Eberl and Vandamme, 2016](#)). In a recent reclassification of this
77 *Burkholderia* “*sensu lato*” taxonomic group, six new genera (*Paraburkholderia*,
78 *Caballeronia*, *Robbsia*, *Mycetohabitans*, *Pararobbsia* and *Trinickia*) have been
79 proposed next to the *Burkholderia sensu stricto* genus ([Beukes et al., 2017](#); [Dobritsa and](#)
80 [Samadpur, 2016](#); [Sawana et al., 2014](#); [Estrada-de Los Santos et al., 2018](#); [Lin et al.,](#)
81 [2020](#); [Lopes-Santos et al., 2017](#)). *Caballeronia* is also called the Stinkbug-associated
82 Beneficial and Environmental (SBE) group of *Burkholderia*, which is divided into two
83 subgroups, group- α (SBE- α) and group- β (SBE- β).

84 A previous survey of the *Caballeronia* symbionts in stinkbugs revealed a
85 regional trend of infection with species of the two subgroups: SBE- α tends to be
86 detected more in Japanese and American stinkbug species of the Coreoidea ([Acevedo et](#)
87 [al., 2021](#); [Garcia et al., 2014](#); [Hunter et al., 2022](#); [Kikuchi et al., 2005, 2011b](#); [Kuechler](#)
88 [et al., 2016](#); [Ohbayashi et al., 2019b](#); [Olivier-Espejel et al., 2011](#)), while the SBE- β
89 tends to be detected more in European species of the Coreoidea ([Kuchler et al., 2016](#);
90 [Ohbayashi et al., 2019b](#)). However, since only a few insect species have been
91 investigated, and even less species from a wide geographic distribution, it remains
92 unclear whether the regional infection trend is general or not. The mechanism of the

93 regional trend of infection remains unclear, but it could be determined by the region-
94 dependent composition of soil microbiota.

95 The Western conifer seed bug *Leptoglossus occidentalis* (Coreoidea: Coreidae)
96 (Fig. 1A), a notorious pest of conifer forests (Lesieur *et al.* 2014), originates from North
97 America (Heidemann 1910; Koerber 1963). However, this stinkbug is nowadays a
98 serious invading pest worldwide. In 1999, *L. occidentalis* was found in Europe for the
99 first time in Italy (Taylor *et al.* 2001) and its population has been expanding rapidly in
100 recent years to whole Europe (Dusoulier *et al.* 2007; Fent and Kment 2011; Gapon
101 2013; Lesieur *et al.*, 2019; Malumphy *et al.* 2008; van der Heyden, 2019), and other
102 more distant regions such as North Africa (Ben Jamaa *et al.* 2013; Gapon 2015). It has
103 also spread to South America (Faúndez *et al.*, 2017) and Asia (Ahn *et al.* 2013;
104 Ishikawa and Kikuhara 2009). In Japan, *L. occidentalis* was first collected in Tokyo in
105 2008 (Ishikawa and Kikuhara, 2009) and as in Europe, it has rapidly spread into almost
106 all areas of Japan including Tohoku and Kyushu districts (Tsuru *et al.*, 2020). Thus, this
107 cosmopolitan species is ideal to clarify regional effects on the symbiotic association. In
108 this study, we investigated the diversity of *Caballeronia* symbionts of *L. occidentalis*
109 collected in Japan, North America, and Europe in order to confirm whether
110 geographical origin affects the gut symbionts of the conifer bug. Furthermore, symbiont
111 inoculation tests of insects reared in the laboratory with SBE- α and SBE- β symbionts
112 were conducted to analyze in these two *Caballeronia* subgroups gut colonization ability
113 and fitness effect on the host insect.

114

115 **Materials and methods**

116 **Insects**

117 Samples of *L. occidentalis* used in this study are listed in [Table 1](#). For bacterial
118 inoculation tests, *L. occidentalis* was collected in Gif-sur-Yvette, France in 2018, and
119 maintained in cages by feeding on pignolia nuts and distilled water containing 0.05%
120 ascorbic acid (DWA) at 25°C under a long-day regime (16h light, 8h dark).

121 **Symbiont inoculation tests**

122 Reared insects were used for inoculation tests with an SBE- α and an SBE- β strain. We
123 used strain RPE225 ([Kikuchi and Fukatsu, 2014](#)), a GFP (green fluorescent protein)-
124 labeled derivative of *B. insecticola* (*Caballeronia insecticola*) strain RPE64, as a typical
125 strain of the SBE- α clade. RPE64 was isolated from midgut crypts of a Japanese
126 specimen of the bean bug *Riptortus pedestris* (Coreoidea: Alydidae) ([Takeshita et al.,](#)
127 [2018](#)). For the SBE- β strain, we selected *Caballeronia* sp. strain 1876, which was
128 isolated from midgut crypts of *L. occidentalis* collected in Gif-sur-Yvette, France in
129 2016. A GFP-labeled derivative of this SBE- β strain, labelled strain 2482, was
130 constructed by a mini-Tn7 transposon delivery system as described previously ([Kikuchi](#)
131 [and Fukatsu, 2014](#)). Insect inoculation tests with these two GFP strains were performed
132 essentially as described previously for other stinkbug species ([Ohbayashi et al., 2015;](#)
133 [Ohbayashi et al., 2019b](#)). Briefly, the two bacterial strains were pre-cultured in YG
134 (yeast extract and glucose) medium (yeast extract 5.0 g L⁻¹, glucose 4.0 g L⁻¹, and NaCl
135 1.0 g L⁻¹) containing rifampicin 30 μ g/ml overnight at 28 °C and 180 rpm, and 200 μ l of
136 the overnight culture was inoculated into fresh 5ml YG, incubated at 28 °C, 180 rpm for

137 2h, and finally diluted to 10^7 CFU/ml in DWA. The cotton pad with DWA was removed
138 from the rearing container with 2nd instar *L. occidentalis* nymphs, and the nymphs were
139 maintained overnight without water to make them thirsty. The symbiont suspensions,
140 prepared as above, were poured onto new cotton pads and placed into the rearing
141 containers. Aposymbiotic nymphs were obtained by placing a cotton pad soaked with
142 DWA only. Containers were maintained as above until analysis.

143 **Fluorescence microscopy observation**

144 The infection status of the inoculated nymphs was confirmed, based on the detection of
145 GFP signals in dissected midgut crypts of third instar nymphs. Dissections were
146 performed in phosphate-buffered saline (PBS) by using fine forceps and scissors under
147 a fluorescent binocular microscope (Leica, MZ FZ III). Pictures of the dissected
148 midguts were taken by a digital camera (Leica, EC3).

149 **Insect survival**

150 The survival rate of aposymbiotic insects and symbiotic insects infected with either the
151 SBE- α strain RPE225 or the SBE- β strain 2482 was estimated by observing regularly
152 insect rearing populations (n=27, 13 or 20 insects respectively) over time until the last
153 adult emergence in the surviving insects. At each observation, the number of alive and
154 dead insects was scored, as well as the number of emerged adults. The survival rate was
155 analyzed by Fisher's exact test with Bonferroni correction. The developmental time
156 until adulthood in the aposymbiotic insect sample was removed from statistical analysis
157 due to a single surviving insect (n=1), and those in SBE- α and SBE- β inoculated insects
158 were analyzed by Student's *t*-test.

159 **Competition assay**

160 For the competition assay, we used the RFP (red fluorescent protein) strain RPE525
161 (SBE- α), a derivative of *C. insecticola* strain RPE64 (Itoh *et al.*, 2019) and the GFP
162 strain 2482 (SBE- β). Exponential phase cells were suspended in DWA and an inoculum
163 containing 10^7 CFU/ml of both strains was prepared from them. The inoculation of
164 insects with the mixed inoculum was performed as above. At 7 days post inoculation,
165 when the insects became 3rd instar nymphs, the midgut crypts were dissected as above.
166 For microscopy analysis, the midgut crypts were observed under a fluorescent
167 microscope (Nikon, Eclipse 80i). For quantitative determination of the two strains, the
168 midgut crypts were collected in 100 μ l of PBS buffer in 1.5 ml tube and homogenized
169 by a sterilized pestle. The pestle was washed by 400 μ l of PBS buffer. The relative
170 number of symbiont cells of GFP and RFP bacteria in the extracts of the midgut crypts
171 and in the bacterial suspension of the inoculum were analyzed by flow cytometry
172 (Beckman Coulter, Cytoflex).

173 **Identification of gut symbionts of *L. occidentalis***

174 Gut symbionts were isolated from the midgut crypts of *L. occidentalis* individuals
175 collected in Japan and France (Table. 1, Fig. 1) by plating crypt contents on YG agar
176 plates. The identity of growing bacteria was determined by direct sequencing of the 16S
177 rRNA gene, as previously described (Kikuchi *et al.*, 2011a). Since conifer bugs
178 captured in Italy, Spain, USA and Canada were preserved in 100% ethanol, their
179 dissected midgut crypts were subjected to DNA extraction, and clone library analysis of
180 the 16S rRNA gene, as described (Ohbayashi *et al.*, 2019b). Sequences obtained by the

181 bacterial isolation and clone analysis were assembled by ATSQ software ver. 5.2
182 (Software Development, Tokyo, Japan), followed by manual corrections. Then, their
183 most similar bacterial species/strains were identified by BLAST comparison. Sequences
184 showing over 99% identity were assigned to the same operational taxonomical unit
185 (OTU).

186 **Molecular phylogenetic analysis of *L. occidentalis* gut symbionts**

187 Multiple sequence alignment of the 16S rRNA gene was constructed with MAFFT on
188 the EMBL-EBI server (Li *et al.*, 2015). A molecular phylogenetic tree was generated by
189 the maximum likelihood (ML) method with removal of gap-including and ambiguous
190 sites, and with bootstrap analysis (1,000 replicates) in MEGA software version 10.1.8
191 (Kumar *et al.*, 2018; Stecher *et al.*, 2020). We selected the Tamura-Nei model of
192 nucleotide substitutions with gamma distributed and invariant sites (G+I) (Tamura and
193 Nei, 1993).

194 **Nucleotide sequence accession numbers**

195 The nucleotide sequence data of the 16S rRNA gene obtained in this study have been
196 deposited in the DDBJ/EMBL/GenBank public databases with the accession
197 numbers LC713090-LC713209 (Table 1).

198

199 **Results**

200 **Identification of gut symbionts in the midgut crypts of *Leptoglossus occidentalis***

201 To investigate the diversity of gut symbionts in the conifer bug *L. occidentalis*,
202 two methods were used depending on the nature of the insect sample. In the bacterial

203 isolation method, 43 symbionts were isolated from the midgut crypts of 22 individuals
204 collected from respective 4 and 2 populations of Japan and France (Table 1). For the
205 clone library analysis of the 16S rRNA gene, 17 specimens collected in Italy, Spain,
206 USA, and Canada were used, from which 77 sequences were obtained in total (Table 1).
207 The 120 assembled sequences were assigned to 11 OTUs based on the 99% sequence
208 identity threshold (Table S1). Seven OTUs (OTU1–OTU7) were identified to be
209 members of *Caballeronia* by BLAST search. The OTU2 and OTU7 represented
210 respectively 69% and 22% of the total 110 sequences identified as *Caballeronia*,
211 indicating that the OTU2 and OTU7 are the main gut symbionts of *L. occidentalis*.
212 These dominant OTUs were detected in both the bacterial isolation and the cloning
213 method, which suggests that there was no method-related bias in the symbiont
214 identification. The remaining four OTUs (OTU8–OTU11), detected only in four
215 individuals of two insect populations, were identified as *Rickettsia* spp., which is a well-
216 known intracellular secondary symbiont of diverse insects (Kikuchi, 2009). The insect
217 specimen in which *Rickettsia* clones were identified, yielded nevertheless a majority of
218 *Caballeronia* clones (Table S1), indicating that those individuals were also colonized
219 with *Caballeronia* symbionts.

220 **Phylogenetic placement of the *Caballeronia* symbionts**

221 Next, we performed molecular phylogenetic analysis based on the 16S rRNA
222 gene including sequences of the seven *Caballeronia* OTUs of the *L. occidentalis* gut
223 symbionts, type strains of *Burkholderia sensu lato* species (*Burkholderia sensu stricto*,
224 *Paraburkholderia*, and *Caballeronia* species) and previously reported *Caballeronia* gut

225 symbionts of various coreoid insects. Within the *Caballeronia* clade, based on the
226 phylogenetic divergence, four subclades were determined: SBE- α , SBE- β , SBE- γ and
227 Coreoidea-clade (Fig. 2). The most dominant OTU2, detected in specimen collected in
228 all countries, was located in the SBE- β . The SBE- β contained four other OTUs (OTU3,
229 OTU4, OTU5, and OTU6) in addition to OTU2 (Fig. 2). On the other hand, the second
230 most dominant OTU7 was placed in the Coreoidea-clade (Fig. 2). This subclade
231 contains neither environmental isolates nor type species, but formed a monophyletic
232 group with many gut symbionts of Japanese and European coreoid bugs (Fig. 2),
233 suggesting their very specialized nature for symbiosis with stinkbug species. OTU1 was
234 located to the SBE- α clade known as major gut symbionts of Japanese and American
235 coreoid stinkbugs (Fig. 2, Fig. S1).

236 The detection rate of the three subclades (SBE- α , SBE- β , and the Coreoidea-
237 clade) in the world's populations of the conifer bug is summarized in Fig. 3 and Fig. S2
238 (also see Table S2), which is based on the phylogenetic placement of the seven OTUs in
239 the subclades of *Caballeronia* (Fig. 2). Overall, conifer bugs were almost exclusively
240 associated with SBE- β , and SBE- α was scarcely detected (Fig. 3). The Coreoidea-clade
241 occupied one fourth of the populations, frequently detected in Japanese and European
242 populations but not in North American populations of the conifer bug (Fig. 3).

243 **Colonization ability and host fitness effect of SBE- α and SBE- β symbiont strains in** 244 **the midgut crypts of *L. occidentalis***

245 SBE- α species have been found consistently in the midgut crypts of 33
246 stinkbug species of the superfamily Coreoidea in previous studies (Acevedo *et al.*,

247 2021; Garcia *et al.*, 2014; Kikuchi *et al.*, 2011a, 2005; Kuechler *et al.*, 2016; Ohbayashi
248 *et al.*, 2019b; Olivier-Espejel *et al.*, 2011; Hunter *et al.*, 2022). However, in the present
249 study, only one out of 120 symbiont isolates or clones from 39 specimen of *L.*
250 *occidentalis* was SBE- α (Fig. 3; Table S1). To confirm whether SBE- α and SBE- β
251 symbionts are capable of colonizing the midgut crypts of the conifer bug, GFP-labeled
252 strains, *C. insecticola* strain RPE225 (SBE- α) and *Caballeronia* sp. strain 2482 (SBE-
253 β), were inoculated to nymphs of *L. occidentalis*. Both the SBE- α and SBE- β strains
254 were capable of colonizing the midgut crypts of *L. occidentalis* (Fig. 4A-B, D-E), as
255 indicated by the enlarged crypts and by the presence of GFP signal. Moreover, the
256 swollen M4B midgut region, typical for symbiont colonization, also confirmed proper
257 colonization by both strains (Fig. 4A-B). On the other hand, aposymbiotic (uninfected)
258 insects showed small M4 crypts and a narrow M4B region (Fig. 4C, F).

259 Next, we investigated the survival of the aposymbiotic, and SBE- α or SBE- β
260 inoculated conifer bugs. The aposymbiotic bugs showed a high mortality during their
261 development (Fig. 4G). Particularly, most of the insects died during 2nd to 3rd instar
262 (Fig. 4G). In contrast, most nymphs inoculated with SBE- α or SBE- β strains survived
263 and reached adulthood (Fig. 4G; survival percentage [adult/total investigated insects] =
264 3.7% [1/27] in aposymbiotic insects; 100% [13/13] in SBE- α -inoculated insects; 90%
265 [18/20] in SBE- β -inoculated insects). The developmental time from hatching to adult
266 emergence was 46, 39 ± 3 , and 40 ± 3 [mean \pm SD] days in aposymbiotic, SBE- α - and
267 SBE- β -inoculated *L. occidentalis*, respectively (Fig. 4H). The survival rate was
268 significantly different between symbiotic (SBE- α or SBE- β inoculated) versus

269 aposymbiotic insects, while there is no significant difference between SBE- α and SBE-
270 β inoculated *L. occidentalis* in survival rate and developmental time (Fig. 4G, H).

271 On the other hand, a competition assay in which nymphs were inoculated with
272 an equal mixture of the SBE- α and the SBE- β strains, demonstrated that the SBE- β
273 significantly outcompeted the SBE- α in the midgut crypts of *L. occidentalis* (Fig. 5).
274 Together, these results strongly suggest that the *Caballeronia* symbiont makes a very
275 large positive contribution to survival and development of *L. occidentalis*, and that the
276 SBE- α has sufficient ability to colonize midgut crypts to give the same fitness effect to
277 *L. occidentalis* as SBE- β . Nevertheless, microbe-microbe competition in the midgut
278 crypts of *L. occidentalis* could contribute to the observed predominance of the SBE- β .

279

280 **Discussion**

281 This study revealed that, although conifer bug specimens are associated
282 with genetically diverse *Caballeronia* (SBE-*Burkholderia*) symbionts, members of the
283 subclades SBE- β are dominant in all investigated Japanese, European, and North
284 American populations of the conifer bug, and the Coreoidea-clade is also found
285 frequently in Japanese and European insects (Fig. 2, Fig. 3; Table S1, S2). Previous
286 studies found that Japanese and American species of the stinkbug superfamily
287 Coreoidea tend to harbor symbionts belonging to the SBE- α subclade (Kikuchi *et al.*
288 2011a; Ohbayashi *et al.* 2019b), but here we show that in the conifer bug, SBE- α was
289 rarely detected, even in Japanese and American populations (Fig. 3; Table S1, S2).
290 From this broad survey in this cosmopolitan species, we conclude that the infection

291 trend is not affected by geographic origin; therefore, it is more likely determined by
292 selection mechanisms in the host insect.

293 The experimental inoculation tests revealed no difference between SBE- α
294 and SBE- β symbionts in colonization ability and fitness effect on the host bug (Fig. 4).
295 However, the competition assay demonstrated that the SBE- β outcompeted SBE- α in
296 the midgut crypts of *L. occidentalis*, probably resulting in the low detection rate of
297 SBE- α in the conifer bug (Fig. 3; Table S1, S2). The infection specificity between the
298 bean bug host and the *Caballeronia* symbiont was demonstrated to be in large part
299 determined by the native symbiont's colonization competitiveness in the midgut. In co-
300 infection experiments, the *Caballeronia* symbionts always outcompeted in midgut
301 crypts the non-symbiont species *Paraburkholderia* and *Pandoraea* that were
302 nevertheless fully capable of colonizing the crypts in the absence of competition species
303 (Itoh *et al.* 2019). The present study is the first report of competition-based selection in
304 the stinkbug midgut between species of different SBE-clades. The details of the
305 competition-based mechanisms are still unknown and further investigation is needed to
306 better understand it.

307 Among the *Caballeronia* gut symbionts of *L. occidentalis*, the second most
308 dominant OTU7 formed a monophyletic group, the Coreoidea-clade, with gut
309 symbionts of other Coreoid species including *Cletus rusticus*, *Plinactus bicoloripes*,
310 *Hygia lativentris*, *Molipteryx fuliginosa*, *Acanthocoris sordidus* (Kikuchi *et al.*, 2011a),
311 *Coreus marginatus* (Ohbayashi *et al.*, 2019b), and *Dicranocephalus albipes* (Kuechler
312 *et al.*, 2016). The Coreoidea-clade includes no environmental isolates/clones and no

313 named species of *Caballeronia*, but consists of only gut symbionts of the Coreoidea
314 (Fig. 2), strongly suggesting that these symbiont strains are highly specific to the insect
315 group. To date, two strains - one is from *A. sordidus*, and the other is the here described
316 OTU7 clone from *L. occidentalis* - have been isolated as culturable symbionts of this
317 clade. It will be of great interest to unveil in the future genomic and physiological
318 features of these Coreoidea-clade symbionts to clarify why these Coreoidea-clade
319 members are specific to this insect group. From the viewpoint of evolution, the
320 intercontinental infection pattern of the Coreoidea-clade (Fig. 3) is notable. Considering
321 that *L. occidentalis* originated from North America (Heidemann 1910; Koerber 1963)
322 and recently invaded to European and Asian countries, we speculate that *L. occidentalis*
323 was originally associated with SBE- β and may have become symbiotic with the
324 Coreoidea-clade as its distribution expanded.

325 *Caballeronia* symbionts make very large positive contribution to fitness of the
326 conifer bug (Fig. 4G), similarly as shown in other coreoid stinkbugs including *C.*
327 *marginatus*, *L. zonatus*, *L. phyllopus* and *R. pedestris* (Ohbayashi *et al.*, 2019b; Hunter
328 *et al.*, 2022; Kikuchi *et al.*, 2007, 2011a). In *R. pedestris*, our previous transcriptomic
329 study revealed that *Caballeronia* provides the host with essential amino acids and
330 vitamins by recycling host metabolic waste materials such as sulfate, allantoin, and urea
331 (Ohbayashi *et al.*, 2019a). This suggests that the *Caballeronia* symbionts critically
332 complement those essential nutrients lacking in conifer seeds in *L. occidentalis*,
333 resulting in a high mortality in aposymbiotic nymphs in *L. occidentalis*. In contrast,
334 aposymbiotic insects in *R. pedestris* showed retarded growth, small body size, and low

335 fecundity compared with symbiotic insects, but aposymbiotic nymphs are able to
336 develop to adulthood with a high survival rate (Kikuchi *et al.*, 2007; Kikuchi and
337 Fukatsu, 2014). Feeding on soybean seeds with high nutritional value probably provides
338 sufficient, although non-optimal, nutrition for development and survival in
339 aposymbiotic *R. pedestris*. The *Caballeronia* symbionts probably play more important
340 metabolic roles for hosts that are feeding on nutritional-poor non-leguminous plants,
341 like *L. occidentalis*.

342 *Rickettsia* was detected in the USA and Canada populations of *L. occidentalis*
343 (Table S1). Although some members are human pathogens, most members of the genus
344 *Rickettsia* are facultative intracellular symbionts in many arthropods (Perlman *et al.*
345 2006), and these symbionts are maintained by vertical transmission. Since *Rickettsia* is
346 known as a reproductive manipulator that causes male-killing and parthenogenesis in
347 many insects (Perlman *et al.* 2006), a similar function could be taken into account in the
348 North America populations of the conifer seed bug. The bacterial group has rarely been
349 detected from stinkbug species except for only some species of the Miridae (Chang and
350 Musgrave 1970; Caspi-Fluger *et al.* 2014; Dally *et al.* 2020), and a more broad survey is
351 still needed to clarify in how far *Rickettsia* is prevalent in heteropteran insects.

352 The present study on *L. occidentalis* first demonstrated that specificity of the
353 *Caballeronia* symbiont is determined by host species rather than biogeography. The
354 symbiont's competitiveness in the gut symbiotic organ is probably pivotal for the
355 specificity. Additionally, differences between species in fitness effect on host bugs, as
356 shown in *L. zonatus* and *L. phyllopus* (Hunter *et al.*, 2022), may also be involved in the

357 specificity. To identify underpinning mechanisms of the host-symbiont specificity in
358 stinkbugs in more detail, the following two points should be clarified in the future.
359 Worldwide distribution of *Caballeronia* in soil needs to be analyzed because the
360 microbial geography is critical in the animals that are tightly associated with
361 environmentally-transmitted beneficial microorganisms. In addition, more experimental
362 inoculation assays, particularly *in vivo* competition assays, are crucial to confirm and to
363 understand how host-microbe specificity is determined in each stinkbug species.

364

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379

380 **Conflict of Interest**

381 The authors declare that they have no competing interests.

382

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Table 1. Information of insect samples investigated in this study.

Country	State/ Prefecture	Locality	Collection year	Collector	Symbiont detection	Specimens number	Symbiot isolates/ clones number	16S rRNA Accession number
Japan	Kumatomo	Koshi	March, 2021	R. Hara, K. Matsunaga	Isolating	3	3	LC713090 – LC713092
	Yamagata	Yuza	April, 2021	Y. Hatanaka	Isolating	4	4	LC713093 – LC713096
	Akita	Akita	October, 2018	K. Takeshita	Isolating	2	2	LC713097 – LC713098
	Akita	Akita	April, 2021	S. Noriyuki	Isolating	4	4	LC713099 – LC713102
France	Essonne	Gif-sur-Yvette ^a	November, 2016	P. Mergaert	Isolating	3	8	LC713103 – LC713110
	Essonne	Gif-sur-Yvette ^a	November, 2021	G. Lextrait	Isolating	6	22	LC713111 – LC713132
Italy	Piedmont	Alessandria	October 2020	S. Chiesa	Cloning	2	6	LC713133 – LC713138
Spain	Catalonia	Artes	April 2021	S. Lopez Romero	Cloning	3	12	LC713139 – LC713150
USA	Idaho	Lenore	October 2020	S. Cook	Cloning	4	31	LC713151 – LC713181
Canada	Nova Scotia	Vaughan	October 2020	S. Blatt	Cloning	4	13	LC713182 – LC713194
	British Col.	Vernon	October 2020	W. Strong	Cloning	4	15	LC713195 – LC713209

^a The same collection site

557

558

559 **Figures legends**

560 **Fig 1.** The conifer bug *L. occidentalis* and its midgut structure. (A) An Adult of *L.*
561 *occidentalis* on leaves of a pine tree, and (B) its whole midgut structure. The shown bug
562 was reared in the laboratory. Symbiont inoculation was performed by feeding the insect
563 on a soil suspension. A symbiont native in the soil colonizes the midgut crypts (M4).
564 Abbreviations of the midgut sections are the following: M1, midgut first section; M2,
565 midgut second section; M3, midgut third section; M4, midgut fourth section with
566 crypts; M4B, M4 bulb; H, hindgut.

567

568 **Fig 2.** Molecular phylogenetic analysis of *Caballeronia* gut symbionts of the conifer
569 bug *L. occidentalis*. A maximum-likelihood tree was generated based on 1,256 aligned
570 nucleotide sites of the 16S rRNA gene. Numbers at the tree nodes indicate the
571 maximum-likelihood bootstrap values (%) with 1,000 replicates, and bootstrap values of
572 over 50 are shown. We referred to the nucleotide sequence information reported in
573 previous studies of *Caballeronia* gut symbionts of coreoid insects in Japan ([Kikuchi et al., 2011a](#);
574 [Kuechler et al., 2016](#); [Ohbayashi et al., 2019b](#)), in America ([Acevedo et al., 2021](#);
575 [Garcia et al., 2014](#); [Hunter et al., 2022](#); [Olivier-Espejel et al., 2011](#)), and in
576 Europe ([Kuechler et al., 2016](#); [Ohbayashi et al., 2019b](#)). The subtree of SBE- α group is
577 compressed. An uncompressed subtree is shown in [Fig. S1](#). Accession numbers in the
578 DNA database (DDBJ/EMBL/GenBank) are shown in square brackets. *L. occidentalis*
579 gut symbionts are shown in blue color with bold case. Stars: bacterial strains used for
580 symbiont inoculation tests. GS: Gut symbiont.

581

582 **Fig. 3** Relative abundance of SBE- α , SBE- β , and Coreoidea-clade bacteria among gut
583 symbionts of conifer bugs normalized by one OTU per one individual at country levels.
584 Number of investigated insects in each country is shown on the graphs, and the precise
585 numbers are provided in [Table S1](#) and [Table S2](#). The relative abundance at local level is
586 shown in Fig. S2.

587

588 **Fig. 4** *L. occidentalis* infection with SBE- α and SBE- β strains and their host fitness
589 effect. (A-C) Whole midguts of *L. occidentalis* at 3rd instar stage, and (D-F) enlarged
590 image of midgut crypts. A dissected midgut inoculated with (A, D) an SBE- α GFP
591 strain, with (B, E) an SBE- β GFP strain, and without (C, F) any inoculant
592 (aposymbiotic). Abbreviation of midgut section is as shown in [Fig. 1](#). (G) Survival rate
593 of *L. occidentalis* inoculated with SBE- α (blue line, n=13) and SBE- β (green, n=20),
594 and without any inoculant (aposymbiotic: gray, n=27). The survival period indicates the
595 time from hatching to the last adult emergence. A black arrow and dotted line indicate
596 symbiont infections at 6 days post-hatching. Different letters indicate statistically
597 significant differences ($P < 0.0001$, Fisher's exact test with Bonferroni correction). (H)
598 Developmental time from hatching to adulthood in conifer bugs inoculated with SBE- α
599 (blue bar, n=13) and SBE- β (green, n=18), and without any inoculant (aposymbiotic:
600 gray line, n=1). Mean \pm SD is shown. n.s. means no statistically significant difference
601 (Student's *t* test).

602 **Fig. 5** Competition assay of RFP-labeled SBE- α and GFP-labeled SBE- β strains in the
603 midgut crypts of *L. occidentalis*. (A) The midgut crypts of *L. occidentalis* at 7 dpi,
604 infected with an equal mixture of both strains. A merged GFP and RFP image is shown.
605 (B) Relative abundance of GFP and RFP strains at the inoculum and at the midgut
606 crypts at 7dpi (n=10). The abundance of GFP and RFP strains at the midgut crypts is
607 significantly different ($P < 1 \times 10^{-10}$, Student's *t* test). Fluorescent microscopic images
608 and relative abundance of GFP and RFP strains in 10 individual insects are shown in
609 Fig. S3. Note that both strains resulted in 100% infection when used in control mono-
610 infections (Fig. S4).

611

612 **Fig. S1** Uncompressed tree of the SBE- α clade shown in Fig. 2. Several sequences
613 derived from American coreoid insects were excluded from this tree due to a short
614 available sequence length (<1,000 bp), but these sequences were confirmed as SBE- α in
615 the previous phylogenetic analysis ([Garcia et al., 2014](#)). Numbers at the tree nodes
616 indicate the maximum-likelihood bootstrap values (%) with 1,000 replicates, and
617 bootstrap values of over 50 are shown. *L. occidentalis* gut symbionts are shown in blue
618 color with bold case. Star: bacterial strain used for symbiont inoculation test in this
619 study. GS: Gut symbiont.

620

621 **Fig. S2** Relative abundance of SBE- α , SBE- β , and Coreoidea-clade bacteria among gut
622 symbionts of conifer bugs normalized by one OTU per one individual at local level.

623 Number of investigated insects in each city is shown on the graphs, and the precise
624 numbers are provided in [Table S1](#) and [Table S2](#).

625

626 **Fig. S3** Competition assay of RFP-labeled SBE- α and GFP-labeled SBE- β strains in the
627 midgut crypts of 10 individual *L. occidentalis*. (A) Differential interference contrast
628 (DIC) and fluorescence microscopy (GFP and RFP) images of the midgut crypts in 10
629 individual insects. Merged GFP and RFP image of the midgut crypts #1 is used in
630 Fig.5A. (B) Relative abundance of GFP and RFP strains in the inoculum and in the 10
631 individual midgut crypts at 7dpi.

632

633 **Fig. S4** Single infection of (A) RFP-labeled SBE- α and (B) GFP-labeled SBE- β strains
634 in the midgut crypts of *L. occidentalis*. DIC and fluorescent microscopic (RFP or RFP)
635 images of the midgut crypts in 4 individual midgut crypts.

636

637

Fig. 1

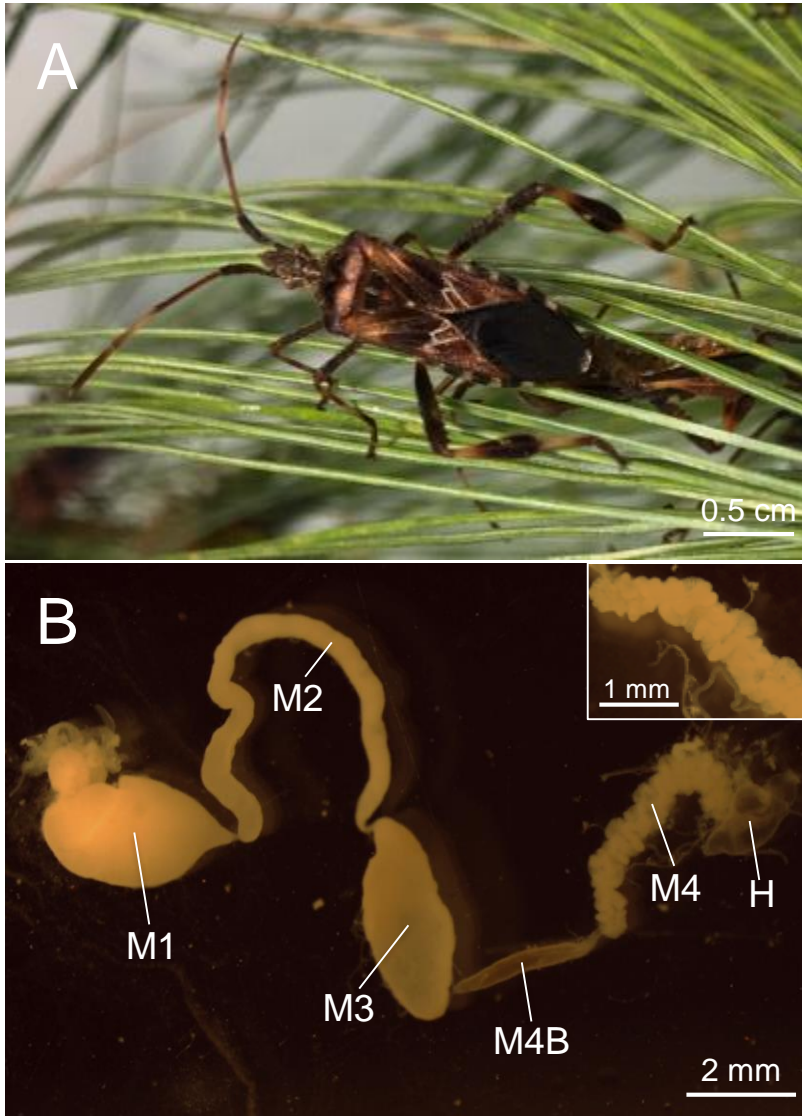


Fig. 2

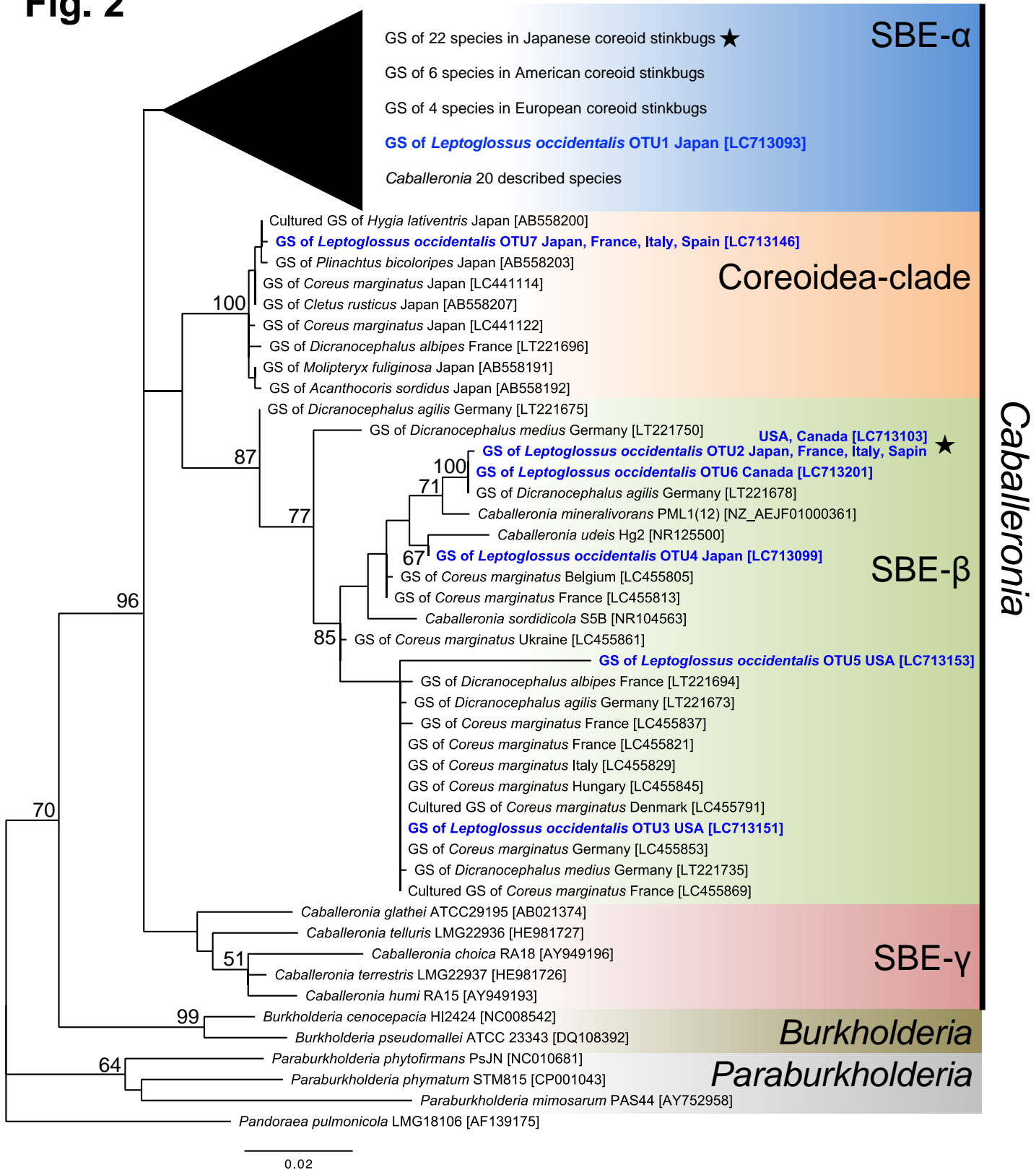


Fig. 3

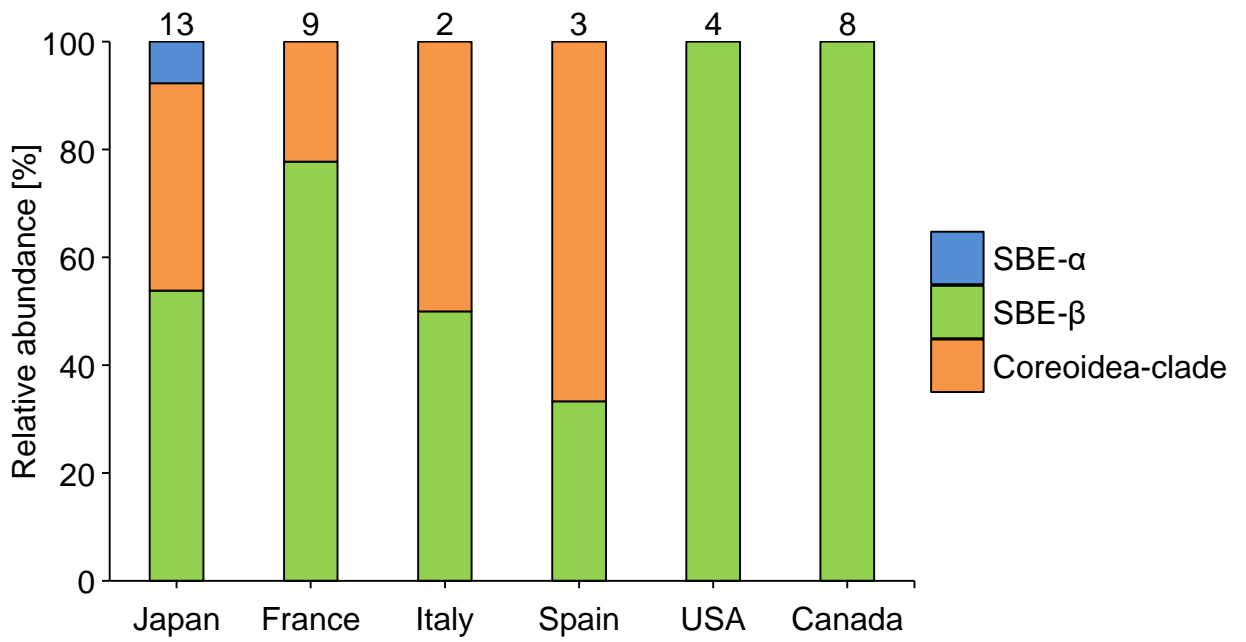


Fig. 4

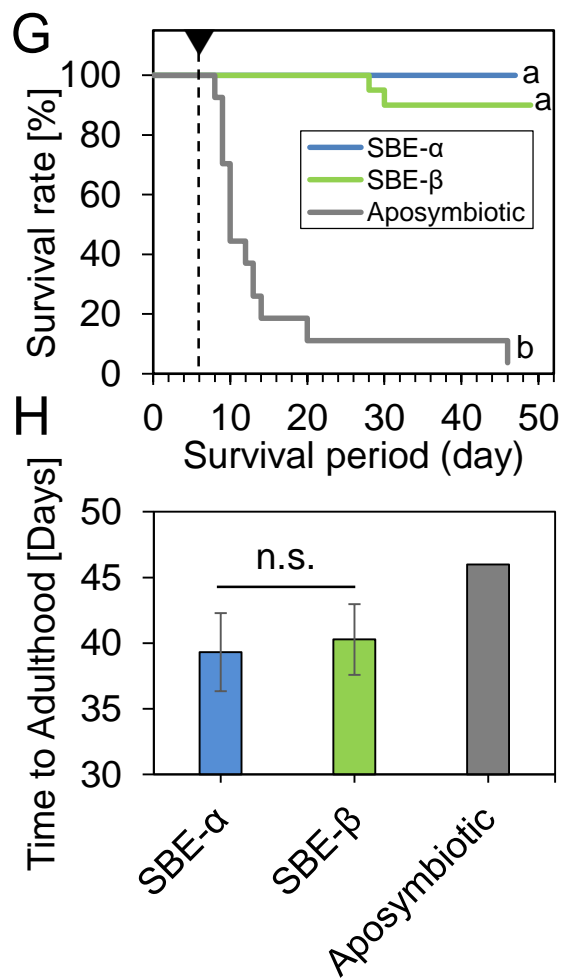
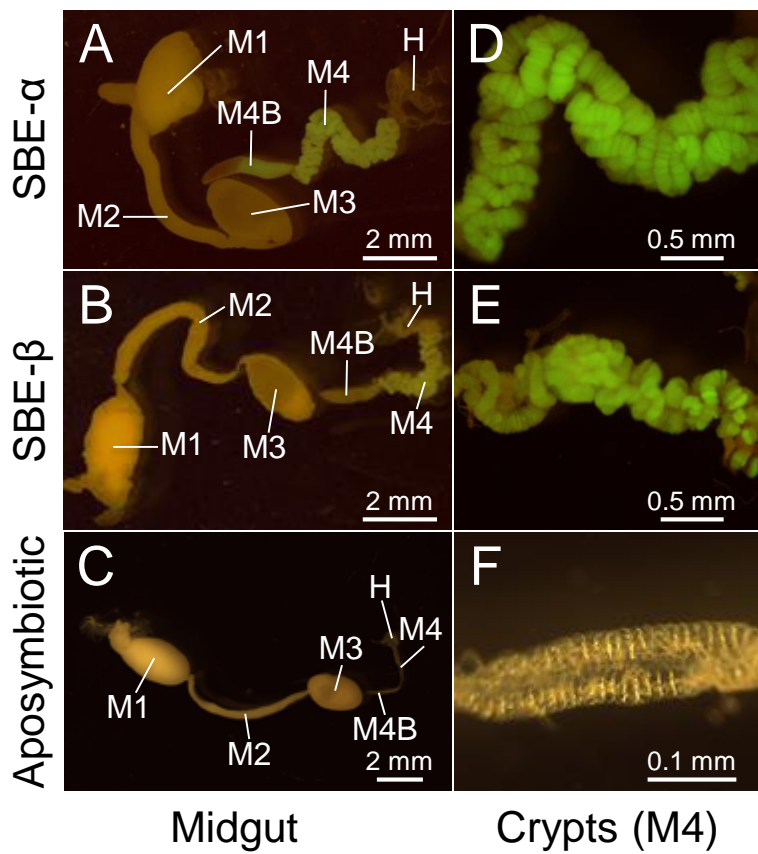


Fig. 5

SBE- α vs SBE- β



B ■ SBE- α (RFP) ■ SBE- β (GFP)

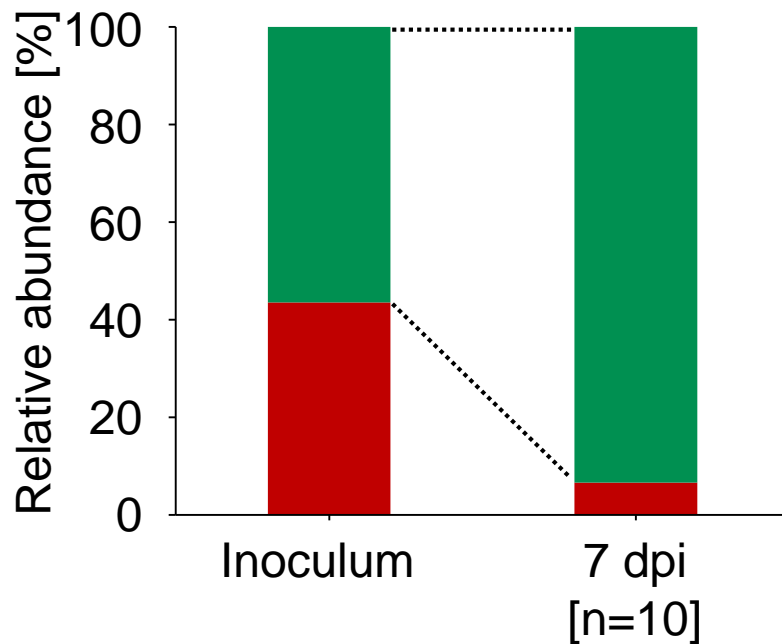


Fig. 5 The competition assay of RFP-labeled SBE- α and GFP-labeled SBE- β strains in the midgut crypts of *L. occidentalis*. (A) The midgut crypts of *L. occidentalis* at 7 dpi. A merged GFP and RFP image is shown. (B) Relative abundance of GFP and RFP strains at the inoculum and at the midgut crypts at 7dpi (n=10). The abundance of GFP and RFP strains at the midgut crypts is significantly different ($P < 1 \times 10^{-10}$, Student's *t* test). Fluorescent microscopic images and relative abundance of GFP and RFP strains in 10 individual insects are shown in Fig. S3.

Fig. S1

SBE- α

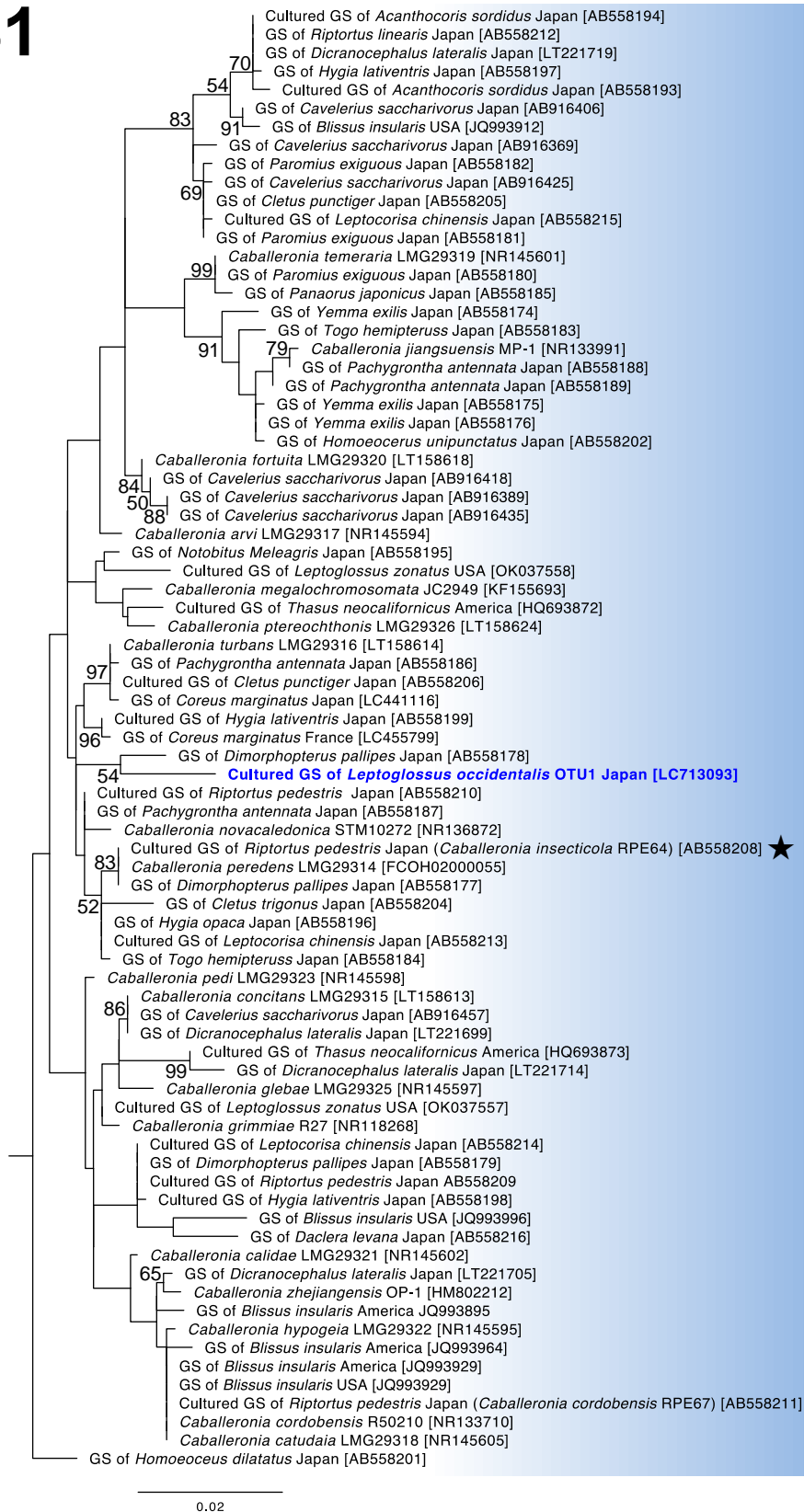


Fig. S1 Uncompressed tree of the SBE- α clade shown in Fig. 2. Several sequences derived from American coreoid insects were excluded from this tree due to a short available sequence length (<1,000 bp), but these sequences were confirmed as SBE- α in the previous phylogenetic analysis (Garcia *et al.*, 2014). Numbers at the tree nodes indicate the maximum-likelihood bootstrap values (%) with 1,000 replicates, and bootstrap values of over 50 are shown. *L. occidentalis* gut symbionts are shown in blue color with bold case. Star: bacterial strain used for symbiont inoculation test in this study. GS: Gut symbiont.

Fig. S2

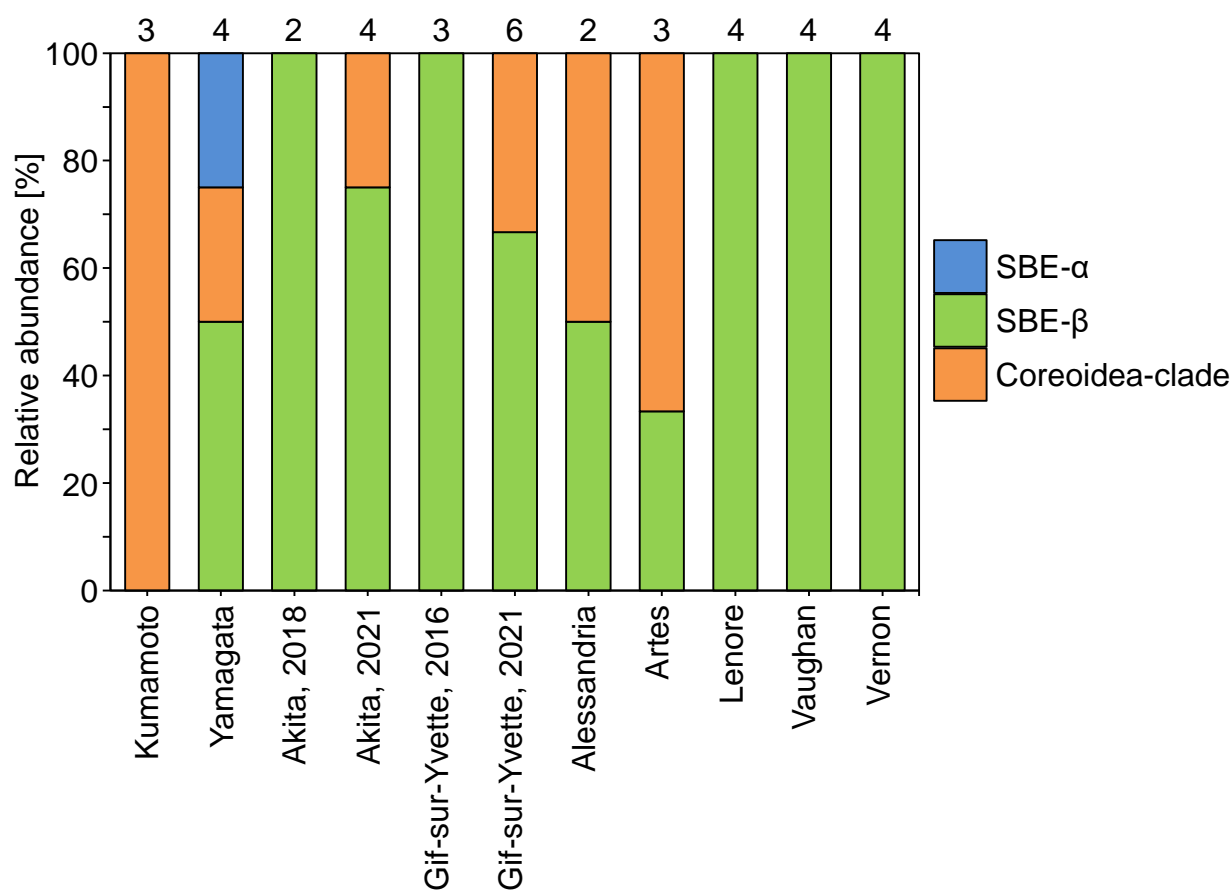
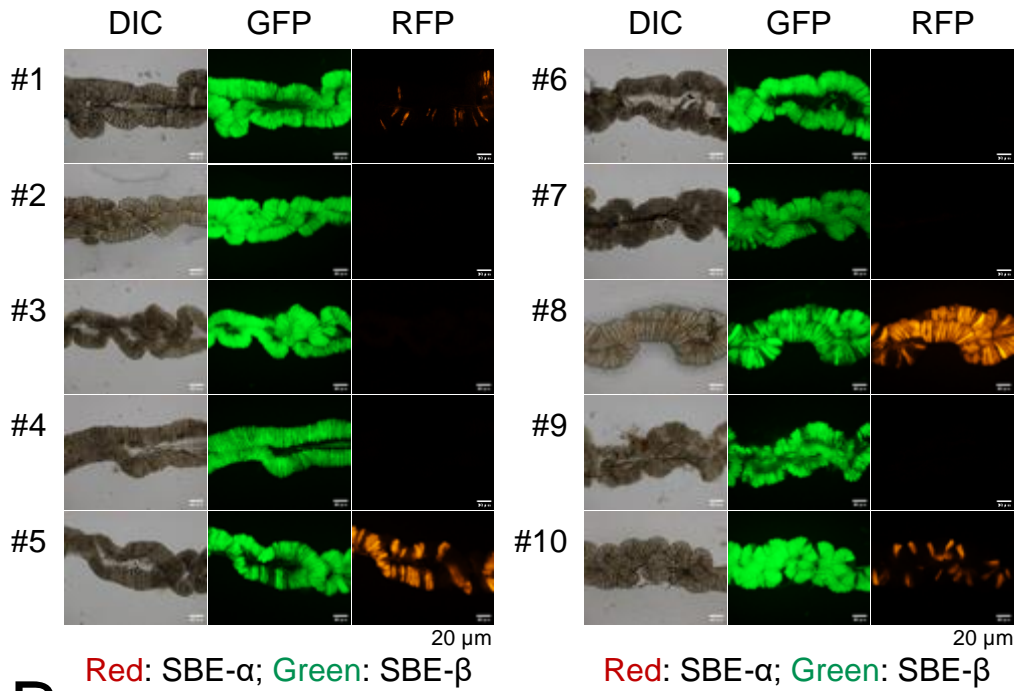


Fig. S2 Relative abundance of SBE- α , SBE- β , and Coreoidea-clade bacteria among gut symbionts of conifer bugs normalized by one OTU per one individual at local level. Number of investigated insects in each city is shown on the graphs, and the precise numbers are provided in [Table S1](#) and [Table S2](#).

Fig. S3

A



B

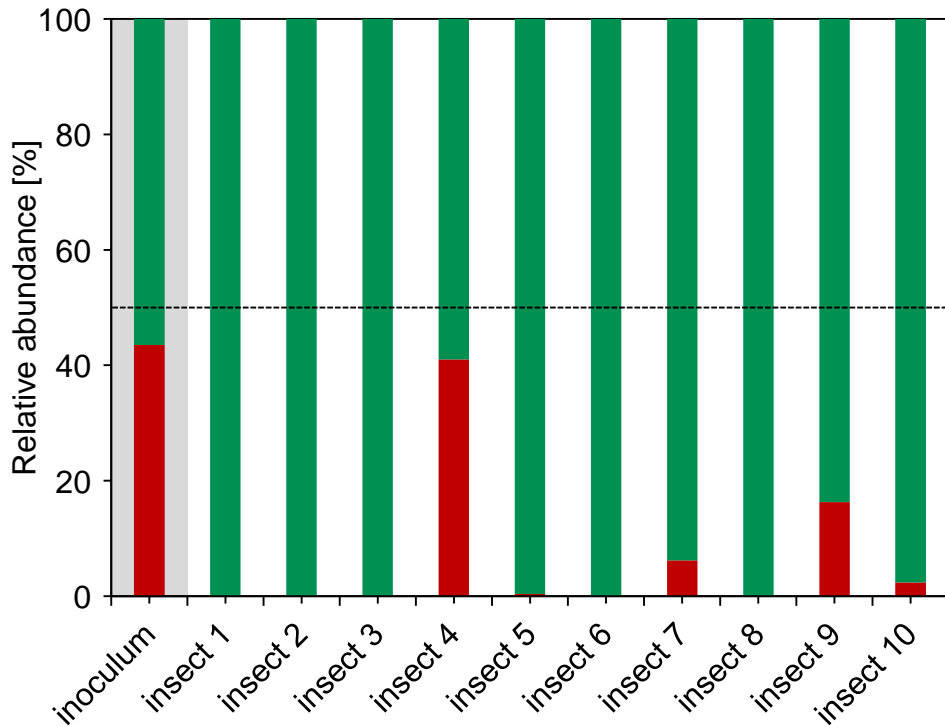


Fig. S3 The competition assay of RFP-labeled SBE- α and GFP-labeled SBE- β strains in the midgut crypts of 10 individual *L. occidentalis*. (A) Differential interference contrast (DIC) and fluorescent microscopic (GFP and RFP) images of the midgut crypts in 10 individual insects. Merged GFP and RFP image of the midgut crypts #1 is used in Fig.5A. (B) Relative abundance of GFP and RFP strains at the inoculum at the 10 individual midgut crypts at 7dpi. Microscopic images and relative abundance data were obtained from 10 insects each.

Fig. S4

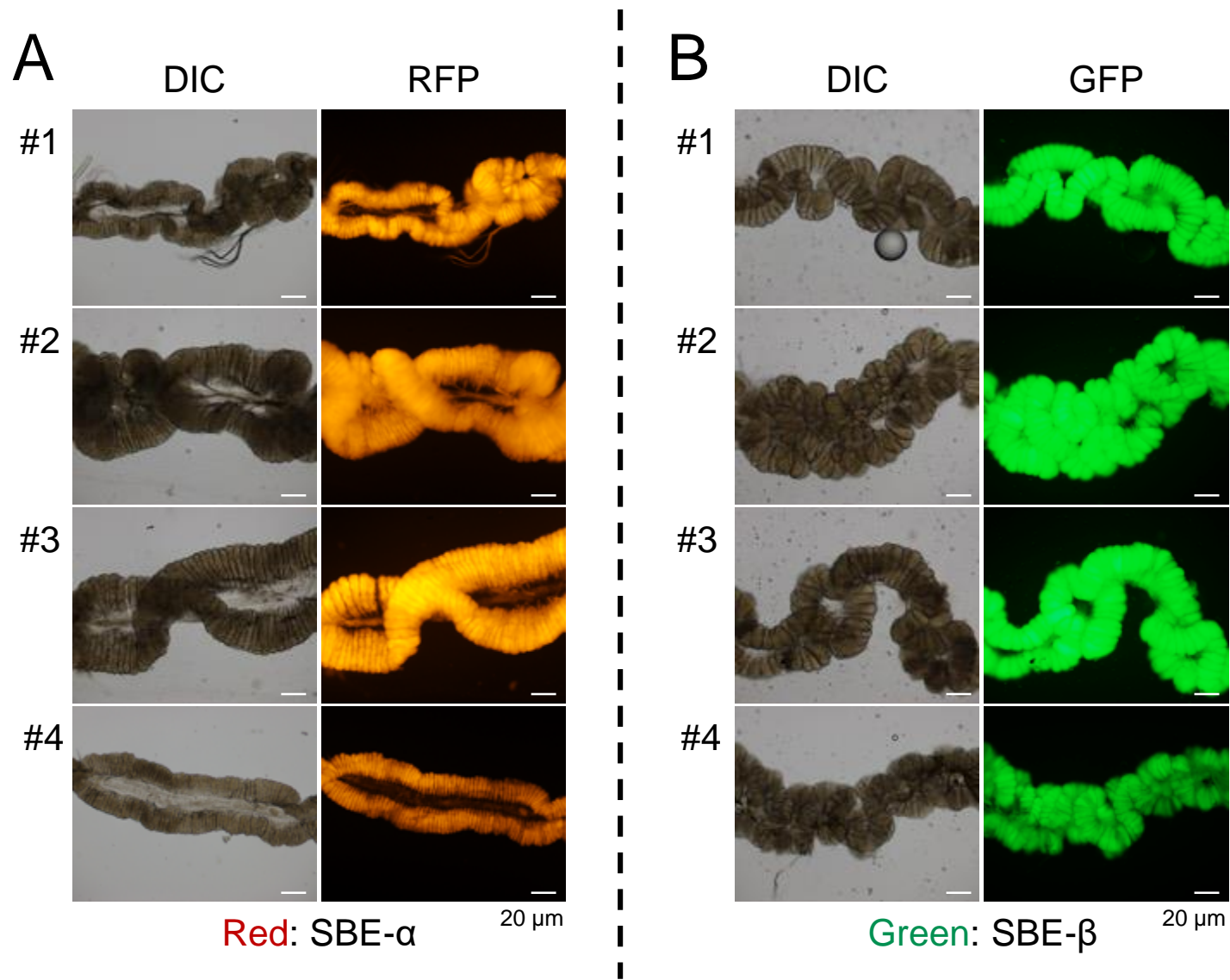


Fig. S4 The single infection of (A) RFP-labeled SBE- α and (B) GFP-labeled SBE- β strains in the midgut crypts of *L. occidentalis*. DIC and fluorescent microscopic (RFP or GFP) images of the midgut crypts in each 4 individual midgut crypts.