Intercontinental diversity of Caballeronia gut symbionts in the conifer pest bug Leptoglossus occidentalis

Tsubasa Ohbayashi, Raynald Cossard, Gaëlle Lextrait, Takahiro Hosokawa, Vincent Lesieur, Kazutaka Takeshita, Kanako Tago, Peter Mergaert, Yoshitomo Kikuchi

Manuscript type: Regular Paper

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

Tsubasa Ohbayashi[1,2*†], Raynald Cossard[2†], Gaëlle Lextrait[2], Takahiro Hosokawa[3], Vincent Lesieur[4], Kazutaka Takeshita[5], Kanako Tago[1], Peter Mergaert[2], and Yoshitomo Kikuchi[6,7]

[1]Institute for Agro-Environmental Sciences, National Agriculture and Food Research Organization (NARO), 305-8604, Tsukuba, Japan

[2]Institute for Integrative Biology of the Cell, UMR9198, CEA, CNRS, Université Paris-Saclay, 91198 Gif-sur-Yvette, France

[3]Department of Biology, Faculty of Science, Kyushu University, 819-0395 Fukuoka, Japan

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

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

[6]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Hokkaido Center, Sapporo 062-8517, Japan

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

[†]These authors contributed equally to this work

* Corresponding author:

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

Abstract (<250 words)

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

Introduction

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

Materials and methods

Insects

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

Symbiont inoculation tests

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

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

Fluorescence microscopy observation

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

Insect survival

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

Competition assay

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

Identification of gut symbionts of *L. occidentalis*

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

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

Molecular phylogenetic analysis of *L. occidentalis* gut symbionts

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

Nucleotide sequence accession numbers

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

Results

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

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

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

Phylogenetic placement of the *Caballeronia* symbionts

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

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

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

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

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

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

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

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

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

Discussion

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

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

Acknowledgements

We thank R. Hara, K. Matsunaga, Y. Hatanaka, S. Noriyuki, S. Chiesa, S. Lopez Romero, S. Cook, S. Blatt, and W. Strong for insect sampling, and J. Lachat, and A. Yokota for assistance with insect rearing. The present work has benefited from Imagerie-Gif core facility supported by l′Agence Nationale de la Recherche (ANR-11-EQPX-0029/Morphoscope, ANR-10-INBS-04/FranceBioImaging ; ANR-11-IDEX-0003-02/Saclay Plant Sciences). This study was supported by a JSPS-CNRS Bilateral Open Partnership Joint Research Project and the CNRS International Research Project "Ménage à Trois" to YK and PM, by the French national research agency (ANR) grant ANR-19-CE20-0007 to PM, and by the JSPS Research Fellowship for Young Scientists (20170267 and 19J01106) to TO, by the the MEXT KAKENHI (18KK0211 to YK and TH; 20H03303 to YK and K. Takeshita), and the Moonshot project JPNP18016, commissioned by the New Energy and Industrial Technology Development Organization (NEDO), to TO and K. Tago.

Conflict of Interest

The authors declare that they have no competing interests.

References

Acevedo, T.S., Fricker, G.P., Garcia, J.R., Alcaide, T., Berasategui, A., Stoy, K.S. and Gerardo, N.M. (2021) The importance of environmentally acquired bacterial symbionts for the squash bug (*Anasa tristis*), a significant agricultural pest. *Front Microbiol* **12**: 719112.

Ahn, S.J., Son, D., Choo, H.Y. and Park, C.G. (2013) The first record on *Leptoglossus occidentalis* (Hemiptera: Coreidae) in Korea, a potential pest of the pinaceous tree species. *J Asia-Pac Entomol* **16**: 281-284.

Ben Jamaa, M., Mejri, M., Naves, P. and Sousa, E. (2013) Detection of *Leptoglossus occidentalis* Heidemann, 1910 (Heteroptera: Coreidae) in Tunisia. *Afr Entomol* **21**: 165-167.

Beukes, C.W., Palmer, M., Manyaka, P., Chan, W.Y., Avontuur, J.R., van Zyl, E., *et al*., (2017) Genome data provides high support for generic boundaries in *Burkholderia* sensu lato. *Front Microbiol* **8**: 1154.

Caspi-Fluger, A., Inbar, M., Mozes-Daube, N., Katzir, N., Portnoy, V., Belausov, E., *et al*., (2012) Horizontal transmission of the insect symbiont *Rickettsia* is plant-mediated. *Proc R Soc B Biol Sci* **279**: 1791-1796.

Chang, K.P. and Musgrave, A.J. (1970) Ultrastructure of rickettsia-like microorganisms in the midgut of a plant bug, *Stenotus binotatus Jak*. (Heteroptera: Miridae). *Can J Microbiol* **16**: 621-622.

Dally, M., Lalzar, M., Belausov, E., Gottlieb, Y., Coll, M. and Zchori-Fein, E. (2020) Cellular localization of two *Rickettsia* symbionts in the digestive system and within the ovaries of the Mirid Bug, *Macrolophous pygmaeus*. *Insects* **11**: 530.

Dobritsa, A.P. and Samadpour, M. (2016) Transfer of eleven species of the genus *Burkholderia* to the genus *Paraburkholderia* and proposal of *Caballeronia* gen. nov. to accommodate twelve species of the genera *Burkholderia* and *Paraburkholderia*. *Int J Syst Evol Microbiol* **66**: 2836-2846.

Dusoulier, F., Lupoli, R., Aberlenc, H.P. and Streito, J.C. (2007) L'invasion orientale de *Leptoglossus occidentalis* en France: bilan de son extension biogéographique en 2007 (Hemiptera Coreidae). *L'Entomologiste* **63**: 303-308.

Eberl, L. and Vandamme, P. (2016) Members of the genus *Burkholderia*: good and bad guys. *F1000Research* **5**: 1007.

Engel, P. and Moran, N.A. (2013) The gut microbiota of insects-diversity in structure and function. *FEMS Microbiol Rev* **37**: 699-735.

Estrada-de Los Santos, P., Palmer, M., Chavez-Ramirez, B., Beukes, C., Steenkamp, E.T., Briscoe, L., *.et al*., (2018) Whole genome analyses suggests that *Burkholderia* sensu lato contains two additional novel genera (*Mycetohabitans* gen. nov., and *Trinickia* gen. nov.): implications for the evolution of diazotrophy and nodulation in the Burkholderiaceae. Genes **9**: 389.

Faúndez, E.I., Rocca, J.R. and Villablanca, J. (2017) Detection of the invasive western conifer seed bug *Leptoglossus occidentalis* Heidemann, 1910 (Heteroptera: Coreidae: Coreinae) in Chile. *Arquivos entomolóxicos*: 317-320.

Fent, M. and Kment, P. (2011) First record of the invasive western conifer seed bug *Leptoglossus occidentalis* (Heteroptera: Coreidae) in Turkey. *North-West J Zool* **7**.

Gapon, D. (2015) First record of *Leptoglossus occidentalis* (Heteroptera: Coreidae) in Morocco. *Heteropterus Rev Entomol* **15**: 161-163.

Gapon, D.A. (2013) First records of the western conifer seed bug *Leptoglossus occidentalis* Heid. (Heteroptera, Coreidae) from Russia and Ukraine, regularities in its distribution and possibilities of its range expansion in the palaearctic region. *Entomol Rev* **93**: 174-181.

Garcia, J.R., Laughton, A.M., Malik, Z., Parker, B.J., Trincot, C., Chiang, S.L., *et al*., (2014) Partner associations across sympatric broad-headed bug species and their environmentally acquired bacterial symbionts. *Mol Ecol* **23**: 1333-1347.

Heidemann, O. (1910) New species of *Leptoglossus* from North America. *Proc Entomol Soc Wash* **12**: 191-197.

Hunter, M.S., Umanzor, E.F., Kelly, S.E., Whitaker, S.M. and Ravenscraft, A. (2022) Development of common leaf-footed bug pests depends on the presence and identity of their environmentally-acquired symbionts. *Appl Environ Microbiol* **88**: e01778-21.

Ishikawa, T. and Kikuhara, Y. (2009) *Leptoglossus occidentalis* Heidemann (Hemiptera: Coreidae), a presumable recent invader to Japan. *Jpn J Entomol* **12**: 115-116.

Itoh, H., Jang, S., Takeshita, K., Ohbayashi, T., Ohnishi, N., Meng, X.Y., *et al*., (2019) Host-symbiont specificity determined by microbe-microbe competition in an insect gut. *Proc Natl Acad Sci U S A* **116**: 22673-22682.

Itoh, H., Tago, K., Hayatsu, M. and Kikuchi, Y. (2018) Detoxifying symbiosis: microbe-mediated detoxification of phytotoxins and pesticides in insects. *Nat Prod Rep* **35**: 434-454.

Kikuchi, Y. (2009) Endosymbiotic bacteria in insects: their diversity and culturability. *Microbes Environ* **24**: 195-204.

Kikuchi, Y. and Fukatsu, T. (2014) Live imaging of symbiosis: spatiotemporal infection dynamics of a GFP-labelled *Burkholderia* symbiont in the bean bug *Riptortus pedestris*. *Mol Ecol* **23**: 1445-1456.

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K. and Fukatsu, T. (2012) Symbiont-mediated insecticide resistance. *Proc Natl Acad Sci U S A* **109**: 8618-8622.

Kikuchi, Y., Hosokawa, T. and Fukatsu, T. (2007) Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Appl Environ Microbiol* **73**: 4308-4316.

463 Kikuchi, Y., Hosokawa, T. and Fukatsu, T. (2011a) An ancient but promiscuous host-
464 symbiont association between *Burkholderia* gut symbionts and their heteropteran
465 hosts. *ISME J* **5**: 446-460.
466 Kikuchi, Y., Hosokawa, T. and Fukatsu, T. (2011b) Specific developmental window for
467 establishment of an insect-microbe gut symbiosis. *Appl Environ Microbiol* **77**:
468 4075-4081.
469 Kikuchi, Y., Meng, X.Y. and Fukatsu, T. (2005) Gut symbiotic bacteria of the genus
470 *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa*
471 *chinensis* (Heteroptera: Alydidae). *Appl Environ Microbiol* **71**: 4035-4043.
472 Kikuchi, Y., Ohbayashi, T., Jang, S. and Mergaert, P. (2020) *Burkholderia insecticola*
473 triggers midgut closure in the bean bug *Riptortus pedestris* to prevent secondary
474 bacterial infections of midgut crypts. *ISME J* **14**: 1627–1638.
475 Koerber, T.W. (1963) *Leptoglossus occidentalis* (Hemiptera, Coreidae), a newly
476 discovered pest of coniferous seed. *Ann Entomol Soc Am* **56**: 229-234.
477 Kuechler, S.M., Matsuura, Y., Dettner, K. and Kikuchi, Y. (2016) Phylogenetically
478 diverse *Burkholderia* associated with midgut crypts of spurge bugs,
479 *Dicranocephalus* spp. (Heteroptera: Stenocephalidae). *Microbes Environ* **31**: 145-
480 153.
481 Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018) MEGA X: molecular
482 evolutionary genetics analysis across computing platforms. *Mol Biol Evol* **35**:
483 1547-1549.
484 Lesieur, V., Lombaert, E., Guillemaud, T., Courtial, B., Strong, W., Roques, A. and
485 Auger-Rozenberg, M.A. (2018) The rapid spread of *Leptoglossus occidentalis* in
486 Europe: a bridgehead invasion. *J Pest Sci* **92**: 189-200.
487 Lesieur, V., Yart, A., Guilbon, S., Lorme, P., Auger-Rozenberg, M.-A. and Roques, A.
488 (2014) The invasive *Leptoglossus* seed bug, a threat for commercial seed crops,
489 but for conifer diversity? *Biological Invasions* **16**: 1833-1849.

Li, W., Cowley, A., Uludag, M., Gur, T., McWilliam, H., Squizzato, S., *et al*., (2015) The EMBL-EBI bioinformatics web and programmatic tools framework. *Nucleic Acids Res* **43**: W580-584.

Lin, Q.H., Lv, Y.Y., Gao, Z.H. and Qiu, L.H. (2020) *Pararobbsia silviterrae* gen. nov., sp. nov., isolated from forest soil and reclassification of *Burkholderia alpina* as *Pararobbsia alpina* comb. nov. *Int J Syst Evol Microbiol* **70**: 1412-1420.

Lopes-Santos, L., Castro, D.B.A., Ferreira-Tonin, M., Correa, D.B.A., Weir, B.S., Park, D., *et al*., (2017) Reassessment of the taxonomic position of *Burkholderia andropogonis* and description of *Robbsia andropogonis* gen. nov., comb. nov. *Antonie Van Leeuwenhoek* **110**: 727-736.

Malumphy, C., Botting, J., Bantock, T. and Reid, S. (2008). Influx of *Leptoglossus occidentalis* Heidemann (Coreidae) in England. *Het News* **12**: 7-9.

Moran, N.A., Ochman, H. and Hammer, T.J. (2019) Evolutionary and ecological consequences of gut microbial communities. *Annu Rev Ecol Evol Syst* **50**: 451-475.

Ohbayashi, T., Futahashi, R., Terashima, M., Barriere, Q., Lamouche, F., Takeshita, K., *et al*., (2019a) Comparative cytology, physiology and transcriptomics of *Burkholderia insecticola* in symbiosis with the bean bug *Riptortus pedestris* and in culture. *ISME J* **13**: 1469-1483.

Ohbayashi, T., Itoh, H., Lachat, J., Kikuchi, Y. and Mergaert, P. (2019b) *Burkholderia* gut symbionts associated with European and Japanese populations of the dock bug *Coreus marginatus* (Coreoidea: Coreidae). *Microbes Environ* **34**: 219-222.

Ohbayashi, T., Takeshita, K., Kitagawa, W., Nikoh, N., Koga, R., Meng, X.Y., *et al.*, (2015) Insect's intestinal organ for symbiont sorting. *Proc Natl Acad Sci U S A* **112**: E5179–E5188.

Olivier-Espejel, S., Sabree, Z.L., Noge, K. and Becerra, J.X. (2011) Gut microbiota in nymph and adults of the giant mesquite bug (*Thasus neocalifornicus*)

(Heteroptera: Coreidae) is dominated by *Burkholderia* acquired de novo every generation. *Environ Entomol* **40**: 1102-1110.

Perlman, S.J., Hunter, M.S. and Zchori-Fein, E. (2006) The emerging diversity of *Rickettsia*. *Proc Biol Sci* **273**: 2097-2106.

Sawana, A., Adeolu, M. and Gupta, R.S. (2014) Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harboring environmental species. *Front Genet* **5**: 429.

Salem, H., Bauer, E., Strauss, A.S., Vogel, H., Marz, M. and Kaltenpoth, M. (2014) Vitamin supplementation by gut symbionts ensures metabolic homeostasis in an insect host. *Proc R Soc Lond B Biol Sci* **281**: 20141838.

Stecher, G., Tamura, K. and Kumar, S. (2020) Molecular Evolutionary Genetics Analysis (MEGA) for macOS. *Mol Biol Evol* **37**: 1237-1239.

Sudakaran, S., Kost, C. and Kaltenpoth, M. (2017) Symbiont acquisition and replacement as a source of ecological innovation. *Trends Microbiol* **25**: 375-390.

Tamura, K. and Nei, M. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* **10**: 512-526.

Takeshita, K. and Kikuchi, Y. (2017) *Riptortus pedestris* and *Burkholderia* symbiont: an ideal model system for insect-microbe symbiotic associations. *Res Microbiol* **168**: 175-187.

Takeshita, K., Tamaki, H., Ohbayashi, T., Meng, X.-Y., Sone, T., Mitani, Y., *et al*., (2018) *Burkholderia insecticola* sp. nov., a gut symbiotic bacterium of the bean bug *Riptortus pedestris*. *Int J Syst Evol Microbiol* **68**: 2370-2374.

Taylor, S.J., Tescari, G. and Villa, M. (2001) A nearctic pest of Pinaceae accidentally introduced into Europe: *Leptoglossus occidentalis* (Heteroptera: Coreidae) in northern Italy. *Entomol News* **112**: 101-103.

545 Tsuru, T.K., Yuito, O. and Akio, T. (2020) First record of alien leaf-footed bug,
546 *Leptoglossus occidentalis* (Insecta, Hemiptera, Coreidae) from Tottori Prefecture,
547 western Japan, with remarks on its range expansion in Japan. *Bulletin of the*
548 *Tottori Prefectural Museum* **57**: 37-43.
549 van der Heyden, T. (2019) Summarized data on the European distribution of
550 Leptoglossus occidentalis Heidemann (Heteroptera: Coreidae: Coreinae:
551 Anisoscelini). *Rev Chil Entomol* **45**: 499-502.
552 Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., *et al*., (1992)
553 Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus
554 *Pseudomonas* homology group II to the new genus, with the type species
555 *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol*
556 *Immunol* **36**: 1251-1275.

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]	Novenber, 2016	P. Mergaert	Isolating	3	8	LC713103 – LC713110
	Essonne	Gif-sur-Yvette [a]	Novenber, 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*
574 *al*., 2011a; Kuechler *et al*., 2016; Ohbayashi *et al*., 2019b), in America (Acevedo *et al*.,
575 2021; 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.

Fig. 3 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 country levels. Number of investigated insects in each country is shown on the graphs, and the precise numbers are provided in Table S1 and Table S2. The relative abundance at local level is shown in Fig. S2.

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

Fig. 5 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, infected with an equal mixture of both strains. 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 ($P < 1x10^{-10}$, Student's *t* test). Fluorescent microscopic images and relative abundance of GFP and RFP strains in 10 individual insects are shown in Fig. S3. Note that both strains resulted in 100% infection when used in control mono-infections (Fig. S4).

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 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.

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

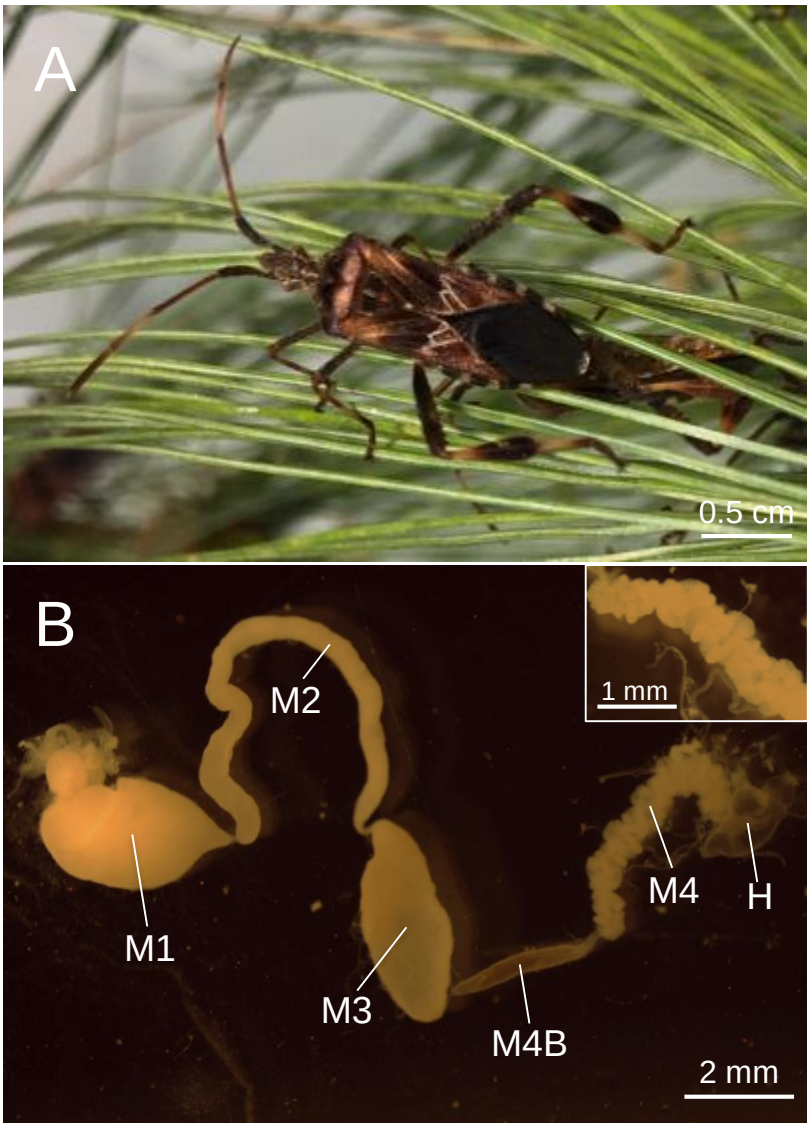

Fig. 2

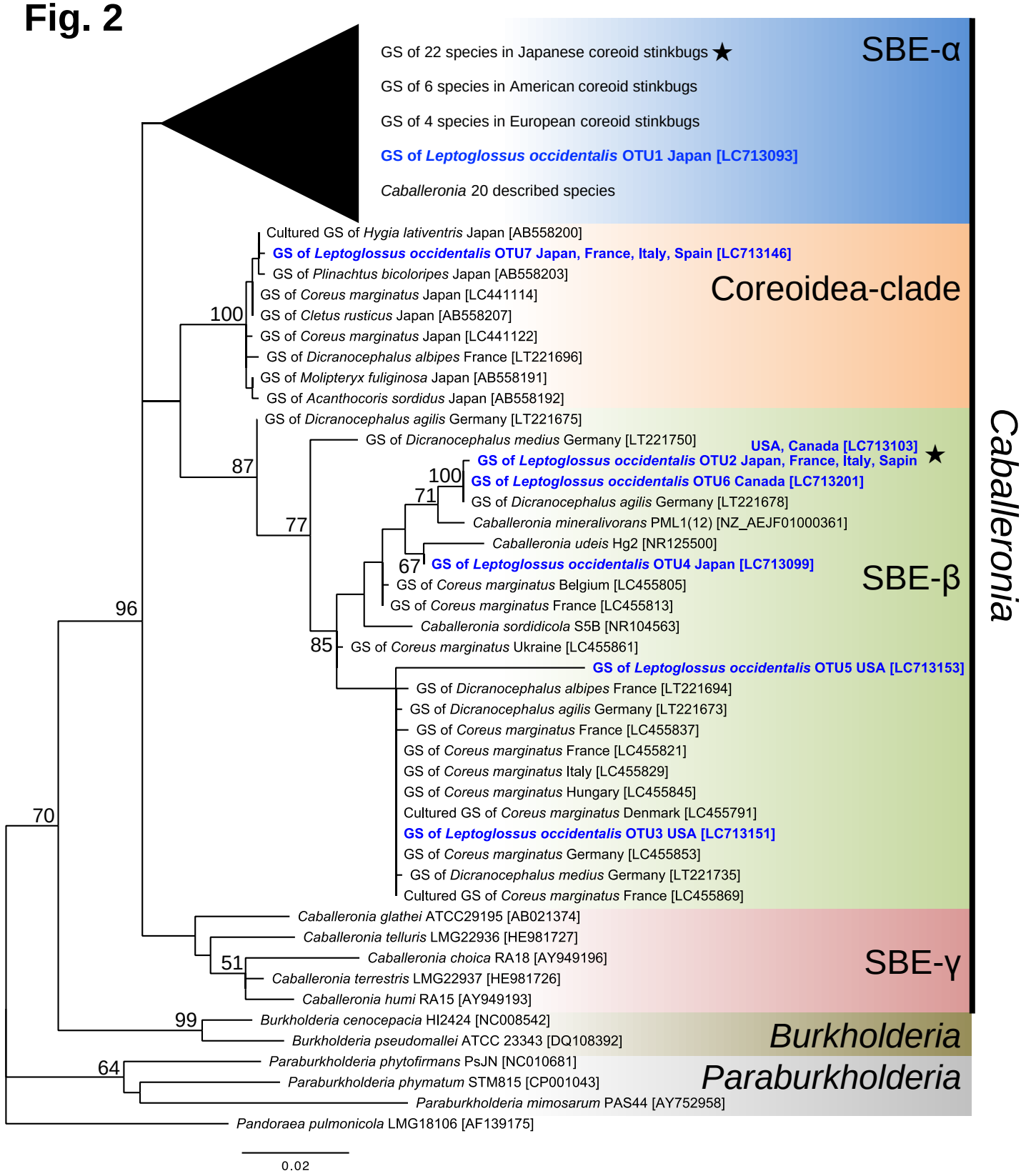
GS of 22 species in Japanese coreoid stinkbugs ★
GS of 6 species in American coreoid stinkbugs
GS of 4 species in European coreoid stinkbugs
GS of Leptoglossus occidentalis OTU1 Japan [LC713093]
Caballeronia 20 described species
SBE-α
Cultured GS of Hygia lativentris Japan [AB558200]
GS of Leptoglossus occidentalis OTU7 Japan, France, Italy, Spain [LC713146]
GS of Plinachtus bicoloripes Japan [AB558203]
GS of Coreus marginatus Japan [LC441114]
GS of Cletus rusticus Japan [AB558207]
GS of Coreus marginatus Japan [LC441122]
GS of Dicranocephalus albipes France [LT221696]
GS of Molipteryx fuliginosa Japan [AB558191]
GS of Acanthocoris sordidus Japan [AB558192]
Coreoidea-clade
GS of Dicranocephalus agilis Germany [LT221675]
GS of Dicranocephalus medius Germany [LT221750]
USA, Canada [LC713103] ★
GS of Leptoglossus occidentalis OTU2 Japan, France, Italy, Sapin
GS of Leptoglossus occidentalis OTU6 Canada [LC713201]
GS of Dicranocephalus agilis Germany [LT221678]
Caballeronia mineralivorans PML1(12) [NZ_AEJF01000361]
Caballeronia udeis Hg2 [NR125500]
GS of Leptoglossus occidentalis OTU4 Japan [LC713099]
GS of Coreus marginatus Belgium [LC455805]
GS of Coreus marginatus France [LC455813]
Caballeronia sordidicola S5B [NR104563]
GS of Coreus marginatus Ukraine [LC455861]
SBE-β
GS of Leptoglossus occidentalis OTU5 USA [LC713153]
GS of Dicranocephalus albipes France [LT221694]
GS of Dicranocephalus agilis Germany [LT221673]
GS of Coreus marginatus France [LC455837]
GS of Coreus marginatus France [LC455821]
GS of Coreus marginatus Italy [LC455829]
GS of Coreus marginatus Hungary [LC455845]
Cultured GS of Coreus marginatus Denmark [LC455791]
GS of Leptoglossus occidentalis OTU3 USA [LC713151]
GS of Coreus marginatus Germany [LC455853]
GS of Dicranocephalus medius Germany [LT221735]
Cultured GS of Coreus marginatus France [LC455869]
Caballeronia glathei ATCC29195 [AB021374]
Caballeronia telluris LMG22936 [HE981727]
Caballeronia choica RA18 [AY949196]
Caballeronia terrestris LMG22937 [HE981726]
Caballeronia humi RA15 [AY949193]
SBE-γ
Burkholderia cenocepacia HI2424 [NC008542]
Burkholderia pseudomallei ATCC 23343 [DQ108392]
Burkholderia
Paraburkholderia phytofirmans PsJN [NC010681]
Paraburkholderia phymatum STM815 [CP001043]
Paraburkholderia mimosarum PAS44 [AY752958]
Paraburkholderia
Pandoraea pulmonicola LMG18106 [AF139175]
100
87
100
71
77
67
96
85
70
51
99
64
0.02
Caballeronia

Fig. 3

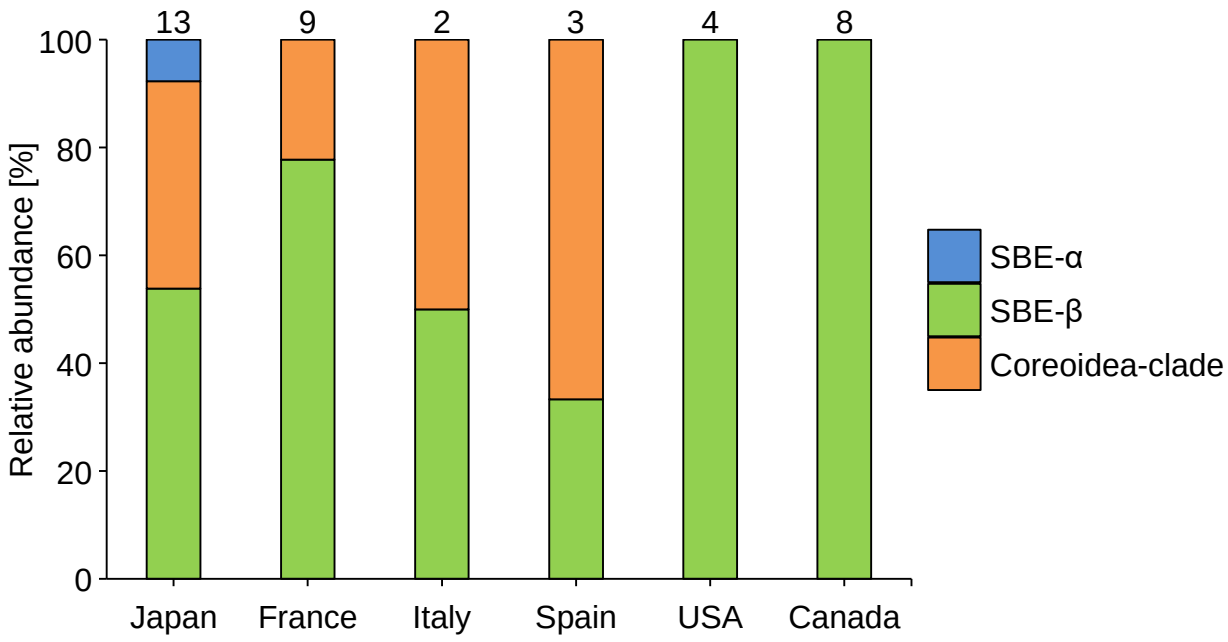
13
9
2
3
4
8
100
80
60
40
20
0
Relative abundance [%]
Japan
France
Italy
Spain
USA
Canada
SBE-α
SBE-β
Coreoidea-clade

Fig. 4

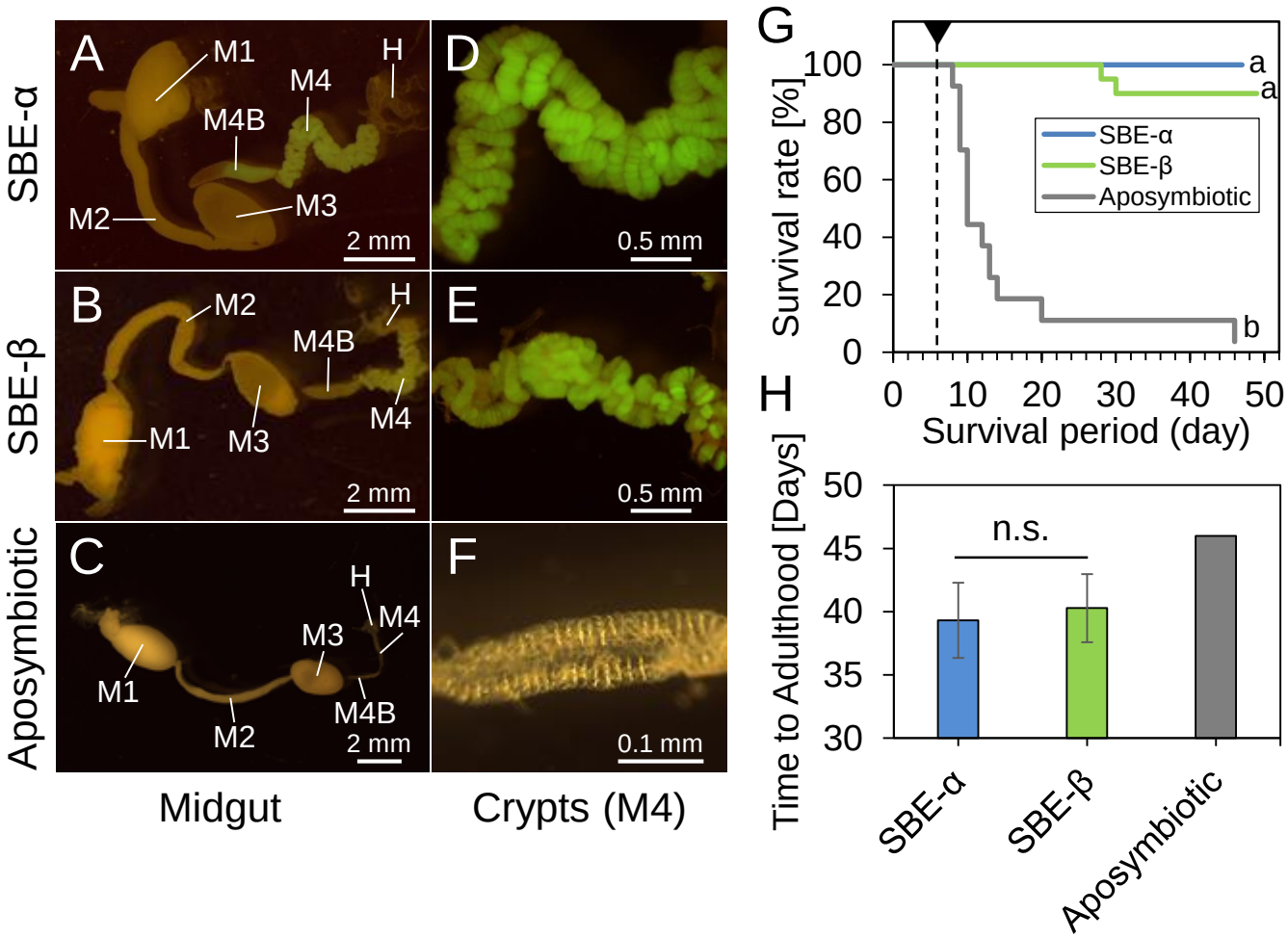
SBE-α
SBE-β
Aposymbiotic
A
M1
H
M4
M4B
M2
M3
2 mm
D
0.5 mm
B
M2
H
M4B
M4
M1
M3
2 mm
E
0.5 mm
C
H
M4
M3
M1
M4B
M2
2 mm
F
0.1 mm
Midgut
Crypts (M4)
G
Survival rate [%]
100
80
60
40
20
0
a
a
b
SBE-α
SBE-β
Aposymbiotic
0
10
20
30
40
50
Survival period (day)
H
Time to Adulthood [Days]
50
45
40
35
30
n.s.
SBE-α
SBE-β
Aposymbiotic

Fig. 5

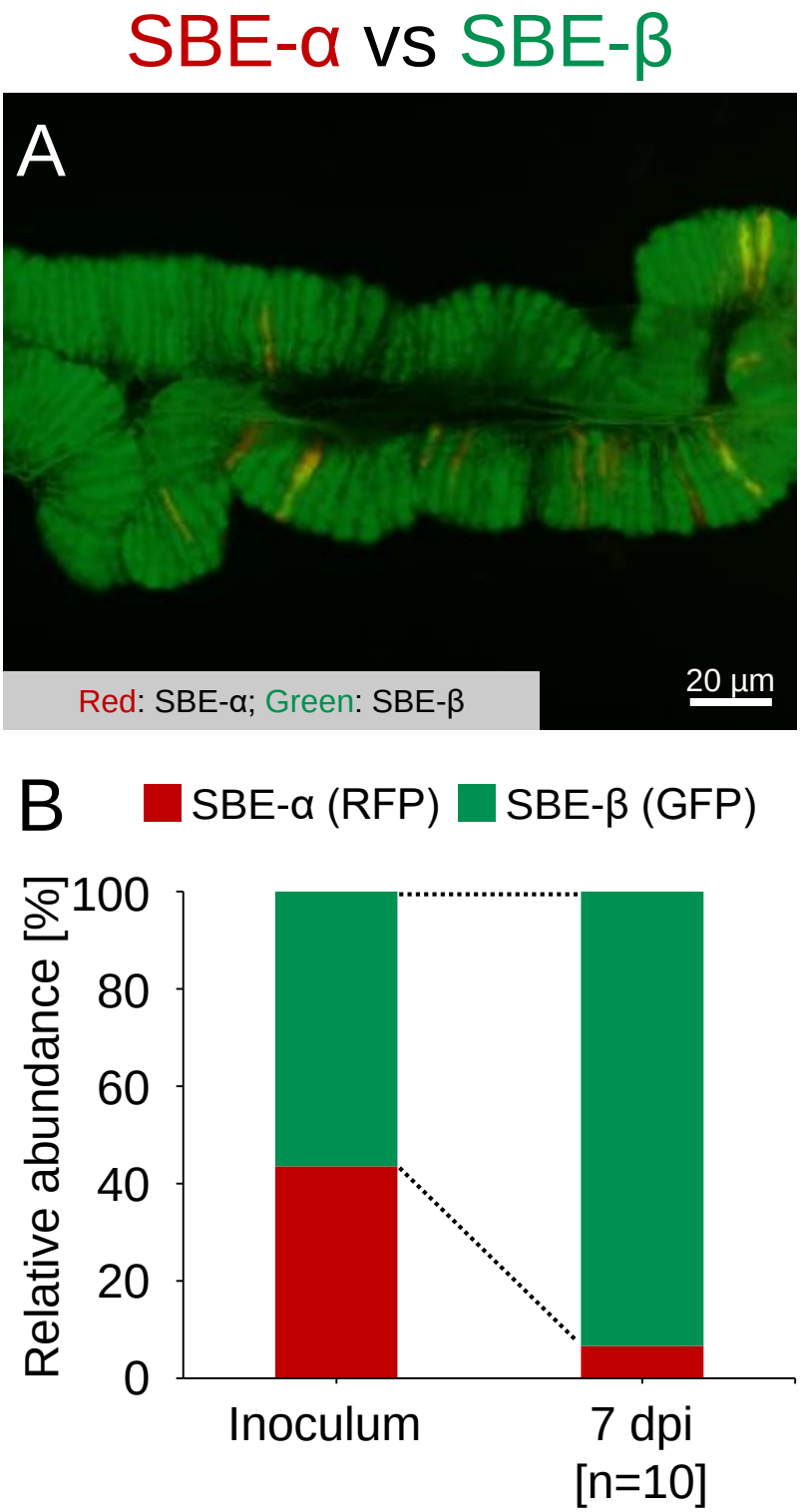

Fig. 5 The competition assay of RFP-labeled SBE-α and GFP-labeled SBE-β strains in the midgut crypts of *L. occidentalis*. (A) The midgut crypts of *L. occidentalis* at 7 dpi. A merged GFP and RFP image is shown. (B) Relative abundance of GFP and RFP strains at the inoculum and at the midgut crypts at 7dpi (n=10). The abundance of GFP and RFP strains at the midgut crypts is significantly different ($P < 1x10^{-10}$, 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

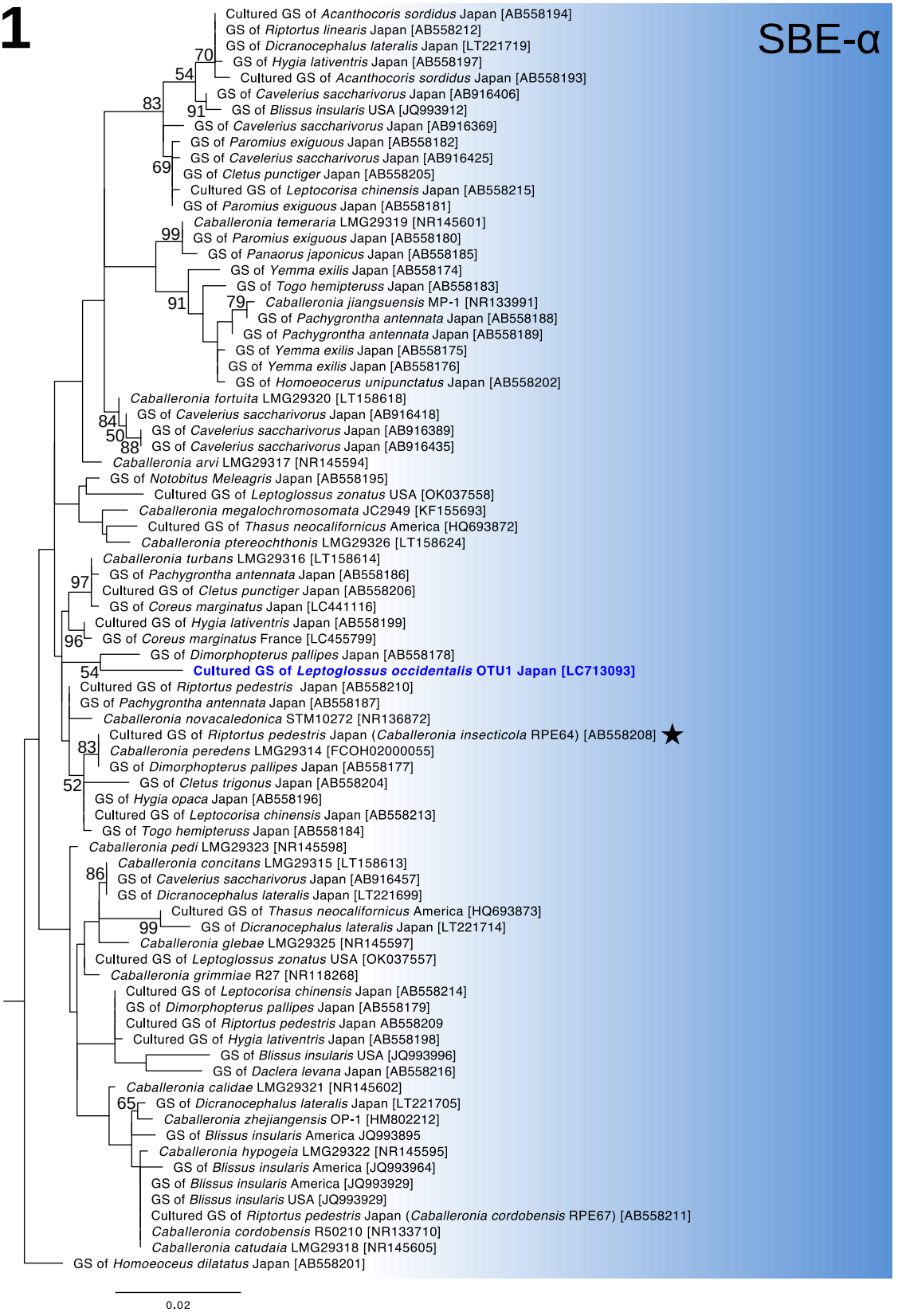

Fig. S1 Uncompressed tree of the SBE-α clade shown in Fig. 2. Several sequences derived from American coreoid insects were excluded from this tree due to a short available sequence length (<1,000 bp), but these sequences were confirmed as SBE-α in the previous phylogenetic analysis (Garcia *et al.*, 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

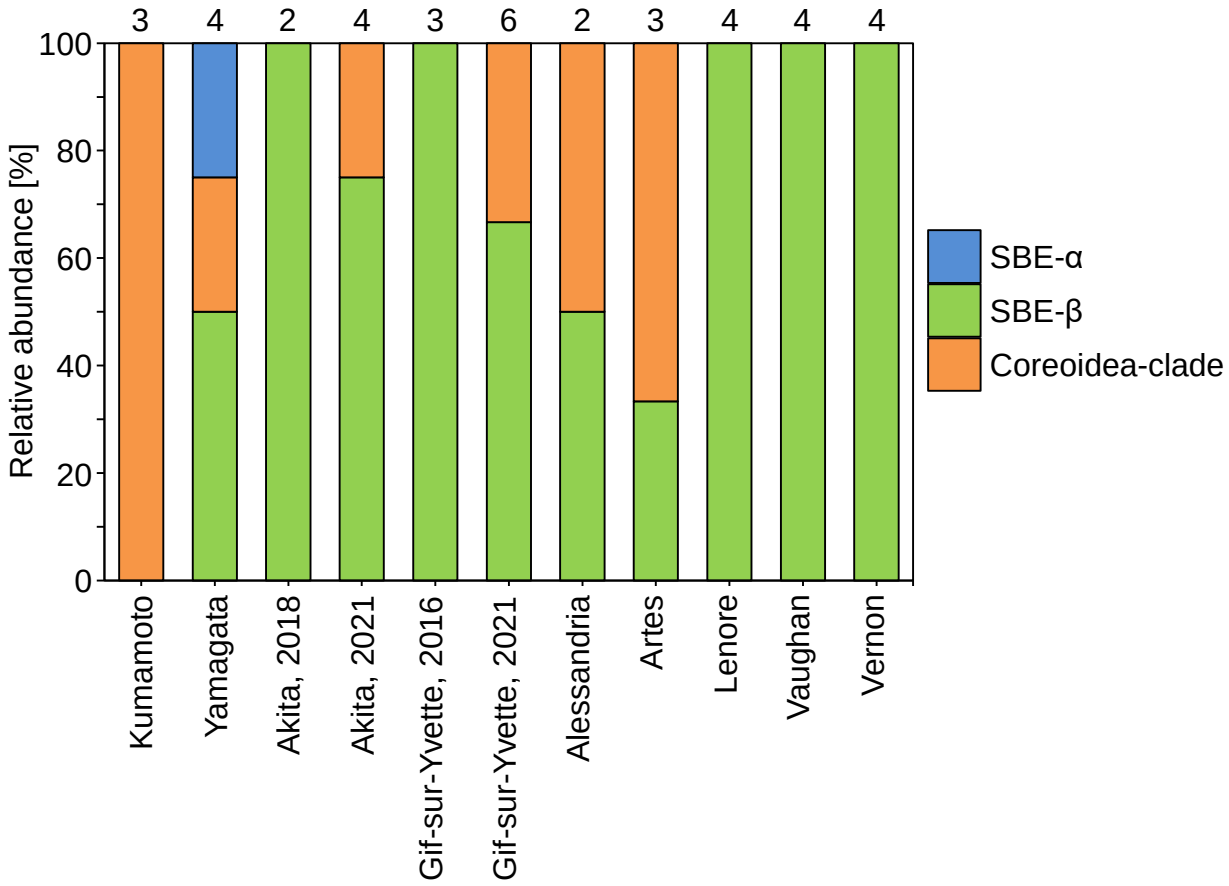

Fig. S2 Relative abundance of SBE-α, SBE-β, and Coreoidea-clade bacteria among gut symbionts of conifer bugs normalized by one OTU per one individual at local level. Number of investigated insects in each city is shown on the graphs, and the precise numbers are provided in Table S1 and Table S2.

Fig. S3

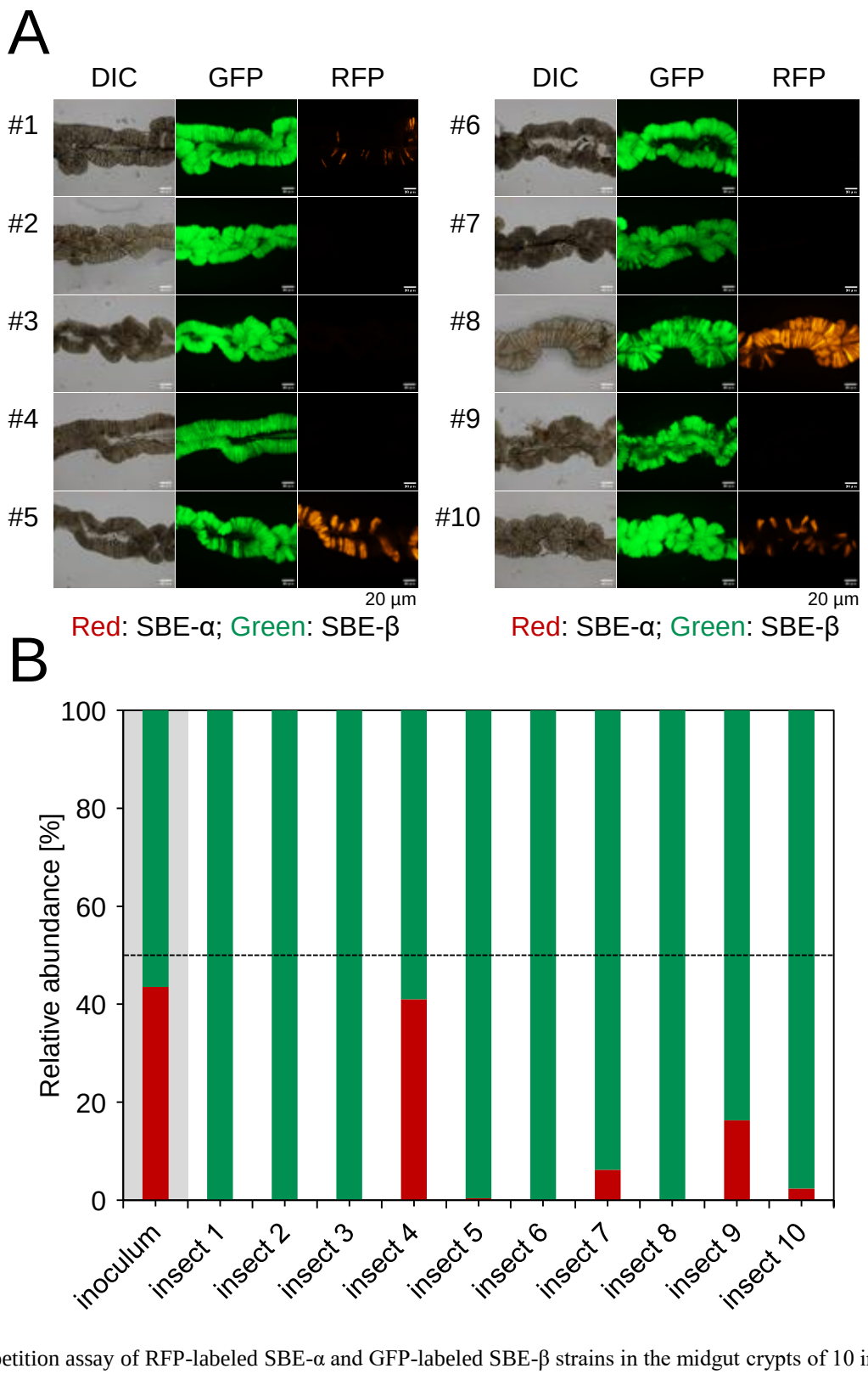

Fig. S3 The competition assay of RFP-labeled SBE-α and GFP-labeled SBE-β strains in the midgut crypts of 10 individual *L. occidentalis*. (A) Differential interference contrast (DIC) and fluorescent microscopic (GFP and RFP) images of the midgut crypts in 10 individual insects. Merged GFP and RFP image 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

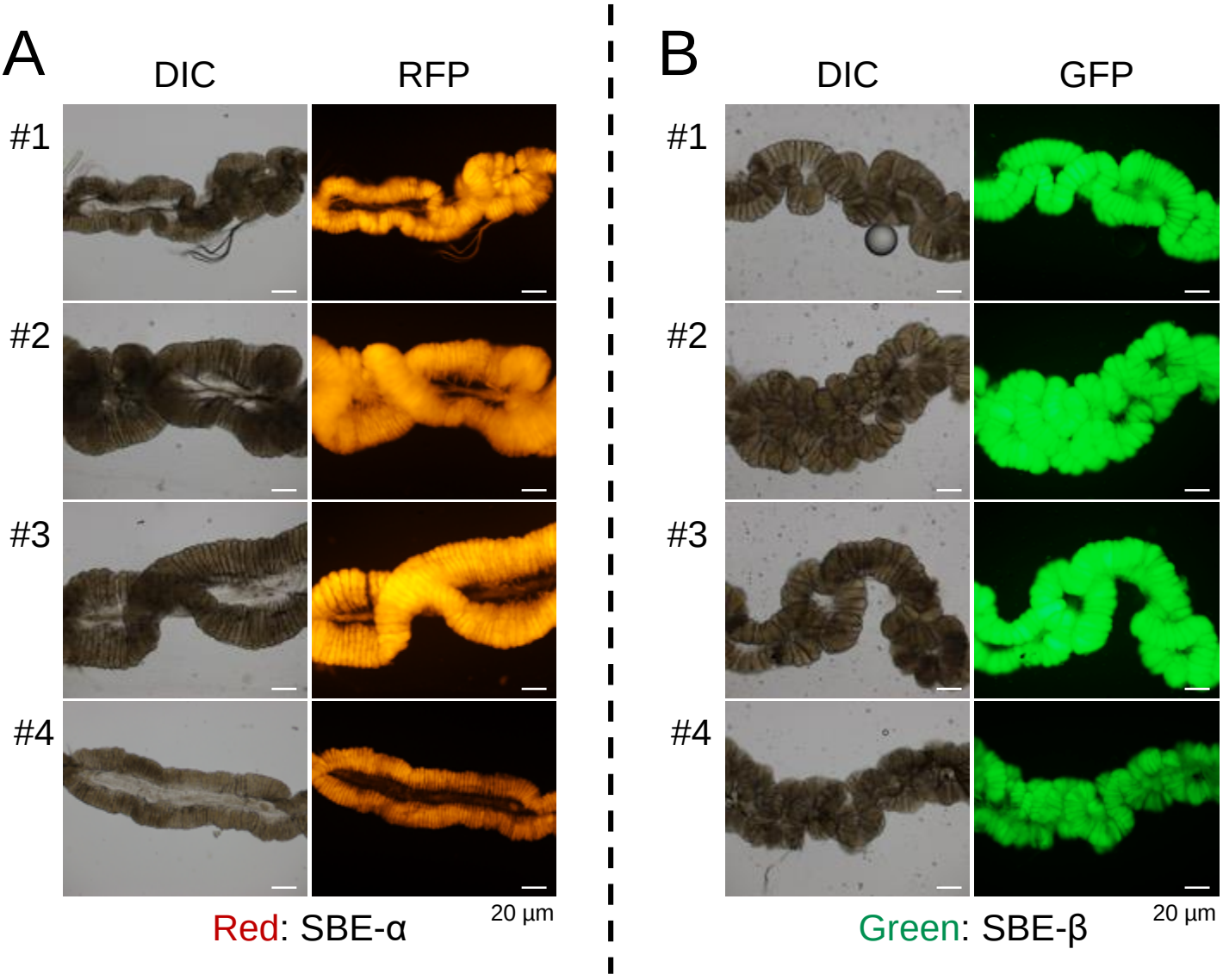

Fig. S4 The single infection of (A) RFP-labeled SBE-α and (B) GFP-labeled SBE-β strains in the midgut crypts of *L. occidentalis*. DIC and fluorescent microscopic (RFP or RFP) images of the midgut crypts in each 4 individual midgut crypts.