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Environmental Acquisition of Gut Symbiotic Bacteria in the Saw-toothed Stinkbug, *Megymenum gracilicorne* (Hemiptera: Pentatomoidea: Dinidoridae)

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Many plant-sucking stinkbugs possess a specialized symbiotic organ with numerous crypts in a posterior region of the midgut. In stinkbugs of the superfamily Pentatomoidea, specific γ-proteobacteria are hosted in the crypt cavities, which are vertically transmitted through host generations and essential for normal growth and survival of the host insects. Here we report the discovery of an exceptional gut symbiotic association in the saw-toothed stinkbug, *Megymenum gracilicorne* (Hemiptera: Pentatomoidea: Dinidoridae), in which specific γ-proteobacterial symbionts are not transmitted vertically but acquired environmentally. Histological inspection identified a very thin and long midgut symbiotic organ with two rows of tiny crypts whose cavities harbor rod-shaped bacterial cells. Molecular phylogenetic analyses of bacterial 16S rRNA gene sequences from the symbiotic organs of field-collected insects revealed that (i) *M. gracilicorne* is stably associated with *Pantoea*-allied γ-proteobacteria within the midgut crypts, (ii) the symbiotic bacteria exhibit a considerable level of diversity across host individuals and populations, (iii) the major symbiotic bacteria represent an environmental bacterial lineage that was reported to be capable of symbiosis with the stinkbug *Plautia stali*, and (iv) the minor symbiotic bacteria also represent several bacterial lineages that were reported as cultivable symbionts of *P. stali* and other stinkbugs. The symbiotic bacteria were shown to be generally cultivable. Microbial inspection of ovipositing adult females and their eggs and nymphs uncovered the absence of stable vertical transmission of the symbiotic bacteria. Rearing experiments showed that symbiont-supplemented newborn nymphs exhibit improved survival, suggesting the beneficial nature of the symbiotic association.

Key words: *Megymenum gracilicorne*, Dinidoridae, Hemiptera, stinkbug, midgut crypts, symbiotic organ, gut symbiont, environmental acquisition, *Pantoea*

INTRODUCTION

Many insects are associated with microbial symbionts, which substantially contribute to physiological, ecological and evolutionary aspects of the host insects (Bourtzis and Miller, 2003; Zchori-Fein and Bourtzis, 2012). Such symbiotic microorganisms are present on the body surface, in the alimentary tract, within the body cavity, or even inside the cells of the host insects (Perlmutter and Bordenstein, 2020; Hosokawa and Fukatsu, 2020). In some cases, the symbiotic microorganisms are indispensable for growth and survival of the host insects, constituting obligatory symbiotic associations and playing important biological roles such as nutrient provisioning (Douglas et al., 2009; Nikoh et al., 2014), food digestion (Brune, 2014; Salem et al., 2017), food production (Vega and Blackwell, 2005; Biedermann and Vega, 2020), etc. In other cases, the symbiotic microorganisms are not essential for survival of the host insects but influential to a variety of host phenotypes in a variety of ways, comprising facultative symbiotic associations and often bringing about beneficial ecological consequences of the host insects such as resistance to parasites and pathogens (Oliver et al., 2003), tolerance to heat stress (Dunbar et al., 2007), resistance to desiccation (Engl et al., 2018), broadening of food plant range (Tsuchida et al., 2004), modifying body color (Tsuchida et al., 2010), affecting reproduc-

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tive mode (Werren et al., 2008), etc. Hence, understanding the diversity and the biological functions of microbial associates of insects is of fundamental importance.

In many groups of plant-sucking stinkbugs (Insecta: Hemiptera), intimate symbiotic associations with gut bacteria are commonly found. In the alimentary tract of these stinkbugs, a posterior region of the midgut is transformed into a specialized symbiotic organ, which develops a number of evaginations or tubular outgrowths, called crypts, for harboring symbiotic bacteria (Glasgow, 1914; Buchner, 1965; Kikuchi et al., 2007, 2011; Hayashi et al., 2015; Oishi et al., 2019). In the superfamily Pentatomoidea, the gut symbiotic bacteria generally belong to the enteric γ-Proteobacteria, vertically transmitted to the offspring via egg surface contamination or deposition of symbiont-containing materials with eggs, and essential for normal growth and survival of the host insects, as demonstrated for such families as the Pentatomidae (Abe et al., 1995; Prado et al., 2006; Prado and Almeida, 2009; Tada et al., 2011; Kikuchi et al., 2012; Bistolas et al., 2014; Hosokawa et al., 2016a, 2016b; Duron and Noël, 2016; Karamipour et al., 2016), the Scutelleridae (Kaiwa et al., 2010; Hosokawa et al., 2019), the Acanthosomatidae (Kikuchi et al., 2009), the Plataspidae (Fukatsu and Hosokawa, 2002; Hosokawa et al., 2006), the Urostylididae (Kaiwa et al., 2014), the Cydnidae (Hosokawa et al., 2012a, 2013), the Parastrachiidae (Hosokawa et al., 2010, 2012b), etc. In the superfamilies Coreoidea and Lygaeoidea, by contrast, the gut symbiotic bacteria generally belong to the genus *Burkholderia* of the β-Proteobacteria, acquired from environmental sources every generation, and beneficial for normal growth and survival of the host insects, as demonstrated for such families as the Alydidae, Coreidae, Stenocephalidae, Blissidae, Berytidae, Pachigronthidae, Rhoparochromidae, etc. (Kikuchi et al., 2005, 2007, 2011; Olivier-Espejel et al., 2011; Boucias et al., 2012; Garcia et al., 2014; Itoh et al., 2014; Kuechler et al., 2016; Ohbayashi et al., 2019).

Whether symbiont transmission occurs vertically, horizontally or environmentally is fundamentally important not only for theoretical prediction but also for empirical understanding of ecological and evolutionary aspects of symbiosis (Ewald, 1987; Kikuchi et al., 2007; Bright and Bulgheresi, 2010; Russell, 2019). In this context, stinkbugs comprise an intriguing group in that a variety of symbiont transmission modes are found in this insect taxon. The above-mentioned previous studies have highlighted the general patterns that γ-proteobacterial gut symbionts are mainly transmitted vertically in the Pentatomoidea whereas β-proteobacterial gut symbionts are usually acquired environmentally in the Coreoidea and Lygaeoidea. However, recently, comprehensive survey of the stinkbug diversity has uncovered notable intermediate cases. For example, in the brown-winged green stinkbug, *Plautia stali* (Pentatomidae), the *Pantoea*-allied gut symbiotic bacteria are vertically transmitted via maternal egg surface contamination and essential for normal host growth, but some symbiont strains are acquired from the soil environments and shared between different stinkbug species and populations in subtropical islands of Japan (Hosokawa et al., 2016a). In the oriental chinch bug, *Cavelerius saccharivorus* (Blissidae), the gut symbiotic bacteria of the genus *Burkholderia* are mainly acquired environmentally, while up to 30% of the nymphal infections may be attributable to maternal transmission via egg surface contamination (Itoh et al., 2014).

The Dinidoridae (Hemiptera: Pentatomoidea) is a relatively small stinkbug family embracing some 17 genera and 100 species in the world (Durai, 1987; Lis, 2006). To our knowledge, there has been no report on the microbial symbionts of the Dinidoridae. Here we investigated the microbial symbiosis in the saw-toothed stinkbug, *Megymenum gracilicorne* (Fig. 1A), the only dinidorid species found in mainland Japan (Ishikawa et al., 2012), and discovered that this species is exceptional among pentatomoid stinkbugs in that its *Pantoea*-allied gut symbiotic bacteria are mainly not transmitted vertically but acquired environmentally.

MATERIALS AND METHODS

Insect material

The samples of *M. gracilicorne* used in this study are as listed (see Supplementary Table S1). The insects were collected from wild cucurbitaceous plants at 11 localities in Japan (see Supplementary Figure S1). Some insects were immediately subjected to experiments, whereas other insects were maintained in the laboratory. The insects were kept at 25°C under constant light in plastic containers (30 cm × 22 cm × 6 cm) in which paper towels, fresh cucumbers as food, and wet cotton pads as water source were provided (Fig. 1B). The insects were transferred to new clean cages every week. Eggs laid on the substrata were collected every week and subjected to rearing and other experiments.

Histology

Adult insects were subjected to dissection of their digestive tract in phosphate buffered saline (PBS: 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4 [pH 7.4]), which consisted of morphologically differentiated midgut regions M1, M2, M3, M4B and M4 (Fig. 1C). The whole digestive tracts were fixed in 4% paraformaldehyde at 4°C overnight and subjected to whole-mount fluorescence in situ hybridization (wFISH) as previously described (Koga et al., 2009), in which Alexa555-labelled oligonucleotide probe EUB338 (5′- Alexa555-GCT GCC TCC CGT AGG AGT -3′) visualized bacterial cells and 4′,6-diamidino-2-phenylindole (DAPI) counterstained host cell nuclei. The tissue specimens were observed under a laser scanning microscope (LSM 700, Zeiss), a fluorescence microscope (DM6B, Leica) or a fluorescence stereomicroscope (M165FC, Leica).

For transmission electron microscopy (TEM), the dissected symbiotic M4 regions were prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 25°C for 1 h, and postfixed with 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) at 4°C for 1 h. Then, the tissues were dehydrated through a water-ethanol series, embedded in Epon812 resin, cut on an ultra-microtome (EM UC7, Leica), mounted on copper meshes, stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (H-7600, Hitachi).

Cultivation and DNA preparation of bacteria in symbiotic midgut region

The midgut M4 region was dissected from each adult insect in PBS, and half of the tissue was ground in 1 ml of PBS in a plastic tube using a plastic pestle. The suspension was appropriately diluted with PBS and subjected to bacterial cell counting using a hemocytometer under a light microscope. The suspension was also serially diluted with PBS to 1/1, 1/100 and 1/10,000, and 50 μl each was spread on an LB agar plate. The inoculated plates were incubated at 25°C under constant darkness for 24 h, and subjected to bacterial colony counting to obtain colony formation unit (CFU) values per M4 sample. For each M4 sample, eight bacterial colonies were picked at random for colony intact PCR of the bacterial 16S

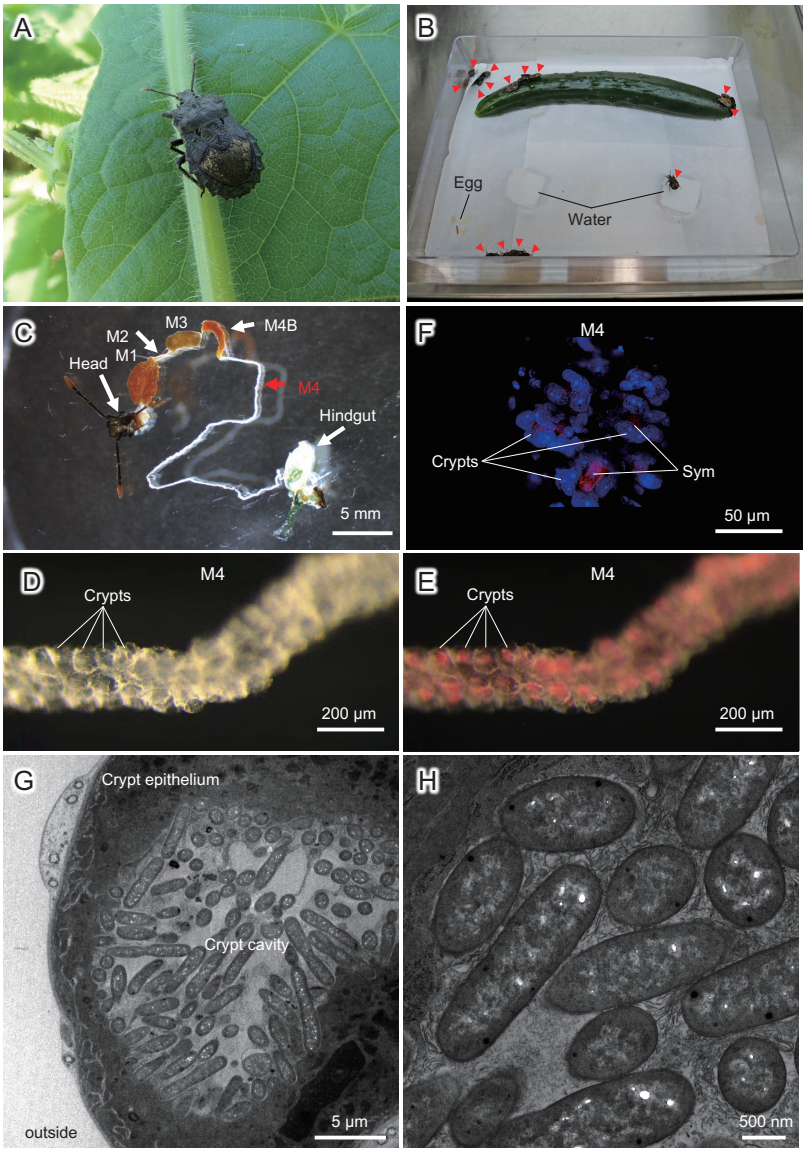

Fig. 1. **(A)** An adult female of *M. gracilicorne* on the vine of *S. angulatus*. **(B)** Laboratory rearing system of *M. gracilicorne* with fresh cucumber and water. Arrowheads indicate the insects. **(C)** Dissected alimentary tract of *M. gracilicorne*. M1, midgut first section; M2, midgut second section; M3, midgut third section; M4B, M4 bulb section; M4, midgut fourth section with crypts (symbiotic organ). **(D)** Bright field image of dissected M4 region, on which many crypts are arranged in two rows. **(E)** Epifluorescence image of dissected M4 region, the same as in **(D),** in which bacterial 16S rRNA is visualized in red by wFISH. Red bacterial signals are localized to the crypt cavities. **(F)** Laser confocal microscopic image of M4 region. Red and blue indicate bacterial 16S rRNA and host nuclear DNA, respectively. Red bacterial signals are seen in the crypt cavities. **(G)** TEM image of an M4 crypt, whose cavity is full of bacterial cells. **(H)** Magnified image of the bacterial cells.

rRNA gene. The other half of the dissected M4 region was directly subjected to DNA extraction using a QIAamp DNA Mini Kit (Qiagen). The DNA samples were used as PCR templates for cloning and sequencing of the bacterial 16S rRNA gene.

PCR, cloning, sequencing and molecular phylogenetic analyses of bacterial 16S rRNA gene

The DNA samples of the dissected M4 regions were subjected to PCR amplification of the bacterial 16S rRNA gene with ExTaq (TaKaRa) and the primers 16SA1 (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 16SB1 (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) (Fukatsu and Nikoh, 1998). The PCR products were subjected to TA-cloning with the T-vector pMD20 (TaKaRa) and chemically competent DH5α cells. Three or more transformant *Escherichia coli* colonies were subjected to intact-colony PCR with AmpliTaq Gold (Thermo Fisher Scientific) and the primers Uni19 (5′-GTT TTC CCA GTC ACG ACG T-3′) and Rev20 (5′-AGC TAT GAC CAT GAT TAC GC-3′). The PCR products were subjected to sequencing with the primers Uni19 and Rev20 by the Sanger method, which was outsourced to Eurofins Genomics. While all the sequences from the same M4 sample were usually identical to each other, minor sequence differences, which were likely due to PCR errors or chimera formation with minor contaminant sequences, were occasionally identified and reconciled by making a consensus.

The bacterial colonies cultured from the dissected M4 regions were individually subjected to intact-colony PCR of the bacterial 16S rRNA gene with AmpliTaq Gold (Thermo Fisher Scientific) and the primers 16SA1 and 16SB1. The PCR products were subjected to nested PCR with ExTaq (TaKaRa) and the primers 16SA2 (5′-GTG CCA GCA GCC GCG GTA ATA C-3′) and 16SB2 (5′-CGA GCT GAC GAC ARC CAT GCA-3′) (Fukatsu and Nikoh, 1998), and direct sequencing with the primer 16SA2.

Multiple alignments of the nucleotide sequences were generated using MUSCLE (Edgar, 2004), from which ambiguously aligned sites and gap-containing sites were removed manually. Maximum-likelihood and neighbor-joining phylogenies were constructed using MEGA X (Kumar et al., 2018) with 500 bootstrap resamplings. Substitution model selection was conducted under the Akaike information criterion using MEGA X. The nucleotide sequences determined in this study were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC594073–LC594427 (see Supplementary Table S1).

Vertical transmission test

Seven field-collected adult females (CKSI20-12–CKSI20-18; see Supplementary Table S1) were individually reared in plastic cages and allowed to lay eggs. The first egg clutch and the second egg clutch were immediately transferred to new, clean, humidified plastic containers, and kept at room temperature. From each of the egg clutches, two 7-day-old eggs and a 7-day-old newborn nymph were collected. In this way, for each of the adult females, a dissected M4 sample, two 7-day-old egg samples, and two 7-day-old nymph samples were obtained and subjected to DNA extraction and PCR amplification of bacterial 16S rRNA gene with ExTaq (TaKaRa) and the primers 16SA2 and 16SB2. The PCR products were analyzed on 1% agarose gels, cloned as described above, and sequenced with the primer Uni19.

Rearing test with or without supplementation of cultured symbiotic bacteria

An isolated and preserved strain of the gut symbiont from an adult female (TSKB13-01; see Supplementary Table S1) was cultured in LB medium at 25°C, washed and suspended in sterilized water, and adjusted to 10^8 CFU/ml. Eggs were collected from mass-reared stocks of *M. gracilicorne* every week, allocated to several

experimental groups (100–400 eggs per group), and kept in clean rearing containers. During the first instar stage, the insects of the symbiont-supplemented groups (19OP + Sym01, 19OP + Sym02, 19OP + Sym03, 20OP + Sym01 and 20OP + Sym02; see Supplementary Table S1) were provided with fresh cucumbers and the symbiont-suspended water, whereas the insects of the control groups (19OP–Sym01, 19OP–Sym02, 19OP–Sym03, 20OP–Sym01 and 20OP–Sym02; see Supplementary Table S1) were provided with fresh cucumbers and sterilized water. Upon second instar molt and thereafter, the insects of both groups were transferred to new rearing cages and provided with fresh cucumbers and sterilized water. During the rearing period of around three months, the rearing cage, food and water were renewed every week, and the insects' molting and survival were monitored and recorded. Survival rates were statistically compared between the symbiont-supplemented groups and the control groups by generalized Wilcoxon test. When all of the insects either reached adulthood or died, eight adult insects were randomly selected from the symbiont-supplemented groups (20OA+Sym01–20OA+Sym08; see Supplementary Table S1) and the control groups (20OA–Sym01–20OA–Sym08; see Supplementary Table S1) and subjected to dissection of their midgut M4 region. The dissected symbiotic organs were subjected to DNA extraction, PCR amplification of the bacterial 16S rRNA gene with the primers 16SA2 and 16SB2, and cloning and sequencing with ExTaq (TaKaRa) and the primer Uni19 as described above.

RESULTS AND DISCUSSION

Histology and localization of gut symbiotic bacteria in *M. gracilicorne*

The dissected digestive tract of *M. gracilicorne* exhibited morphologically differentiated midgut regions M1, M2, M3, M4B and M4 (Fig. 1C), as generally observed in diverse stinkbugs (Kikuchi et al., 2008, 2011; Ohbayashi et al., 2015; Oishi et al., 2019). In comparison with the other stinkbugs, *M. gracilicorne* was peculiar in that the M4 region was very thin and long: thinner than any other midgut regions and longer than the total stretch from M1 to M4B (Fig. 1C). Though inconspicuous, close observation of the M4 region revealed small crypts arranged generally in two rows (and partially in three or four rows) (Fig. 1D), as previously described anatomically (Miyamoto, 1961). FISH detected bacterial 16S rRNA signals localized to the inner cavity of the crypts (Fig. 1E and F). TEM visualized the crypt cavity full of rod-shaped bacterial cells (Fig. 1G and H).

Molecular phylogenetic characterization of gut symbiotic bacteria in field populations of *M. gracilicorne*

The gut symbiotic bacteria of *M. gracilicorne* were characterized by cloning and sequencing of 16S rRNA gene sequences from the dissected M4 regions. In 2013, 13 adult insects from eight localities (blue in Supplementary Figure S1; see Supplementary Table S1) were subjected to the analyses, which consistently yielded identical or very similar 16S rRNA gene sequences belonging to *Pantoea*-allied γ-proteobacteria from all of the insects (blue sequences in Fig. 2). Of these, nine sequences from six localities (Tsukuba, Miyazaki, Kita-Kyushu, Kumamoto, Kagoshima and Chikusei) were completely or nearly identical to each other, and also to the sequence of an environmental bacterium, designated as type X2, which was shown to be potentially capable of symbiosis with the pentatomid stinkbug *Plautia stali* (Hosokawa et al., 2016a); one sequence from Fukuyama clustered with the enteric/environmental bacterium *Leclercia adecarboxylata*; two

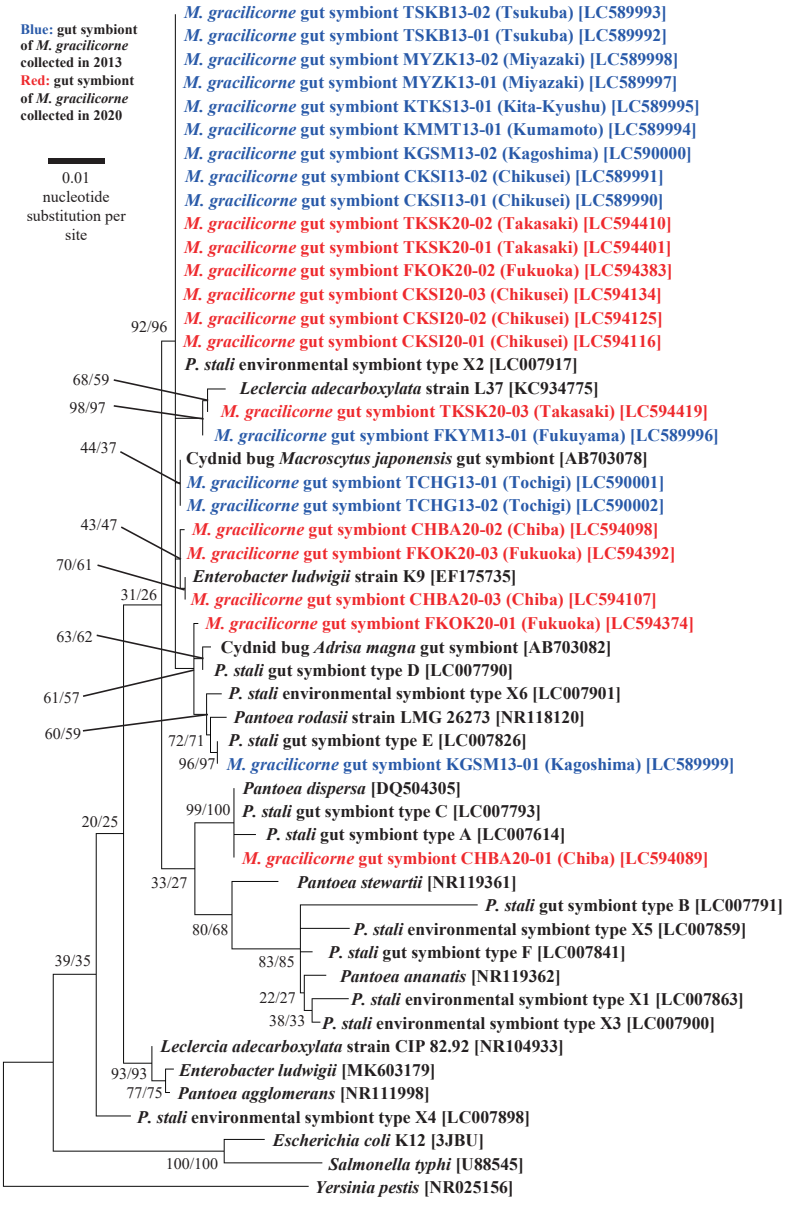

Fig. 2. Molecular phylogenetic placement of the gut symbiotic bacteria of *M. gracilicorne* collected from field populations. Maximum-likelihood phylogeny inferred from 1325 aligned nucleotide sites of the 16S rRNA gene under the GTR+G+I model is shown, while neighbor-joining phylogeny exhibits substantially the same tree topology. Bootstrap probabilities are indicated at the nodes in the order of maximum-likelihood/neighbor-joining. Accession numbers for the sequences are shown in brackets. As for colors, see the notes on the upper left.

sequences from Tochigi were almost identical to the sequence of the gut symbiont of the cydnid stinkbug *Macroscytus japonensis* (Hosokawa et al., 2012a); and one sequence from Kagoshima was identical to the sequence of the cultivable gut symbiont, designated as type E, of the stinkbug *P. stali* (Hosokawa et al., 2016a) (see Fig. 2). In 2020, we also analyzed 12 insects from four localities (red in Supplementary Figure S1; see Supplementary Table S1), and obtained similar results (red sequences in Fig. 2): six sequences from three localities (Takasaki, Fukuoka and Chikusei) were completely or nearly identical to the type X2 potential symbiont of *P. stali*; one sequence from Takasaki was allied to *L. adecarboxylata*; three sequences from Chiba and Fukuoka were allied to *Enterobacter ludwigii*; one sequence from Fukuoka was related to the sequence of the gut symbiont of the cydnid stinkbug *Adrisa magna* (Hosokawa et al., 2012a); and one sequence from Chiba was identical to the sequence of the cultivable gut symbiont, designated as type C, of the stinkbug *P. stali* (Hosokawa et al., 2016a). These results indicated that (i) *M. gracilicorne* is stably associated with *Pantoea*-allied γ-proteobacteria within the crypts of the midgut M4 region, (ii) the symbiotic bacteria exhibit a considerable level of diversity across individuals and populations of *M. gracilicorne*, (iii) the major symbiotic bacteria are the type X2 potential symbiont of *P. stali* of environmental origin, and (iv) the minor symbiotic bacteria also represent other cultivable symbionts of *P. stali* and other stinkbugs.

Cultivability of gut symbiotic bacteria of *M. gracilicorne*

These observations suggested the possibility that the gut symbiotic bacteria of *M. gracilicorne* may be derived from cultivable environmental bacteria. In 2013, we performed a preliminary cultivation trial of homogenized M4 regions, and observed bacterial colony formation on LB agar plates for 10 of 13 insects examined (see Supplementary Table S1). In 2020, we performed more careful and thorough investigation on the cultivability of the symbiotic bacteria using 12 adult females from four localities (Table 1; see Supplementary Table S1 and see Supplementary Table S2). Direct cell counting detected largely constant bacterial titers ranging from 10^7 to 10^8 per M4 region (Table 1). Meanwhile, bacterial culturing on LB agar plates detected CFU values mostly ranging from 10^4 to 10^5 per M4 region (Table 1), indicating that only a very small fraction (~1/1000) of the gut symbiont population may be capable of colony formation on agar plates. Plausibly, the majority of the gut symbiont population may be in a non-proliferating status, and the M4 homogenization must be so damaging that the majority of the symbionts are killed in the experimental treatments. Such low efficiencies of symbiont culturing were also observed among cultivable gut symbiotic bacteria of other stinkbugs (T. Hosokawa et al., unpublished data). Notably, three insects from Takasaki (and one insect from Chikusei) exhibited conspicuously low CFU values of 10^2 to 10^3 per M4 region (Table 1). When eight bacterial colonies from each of the insects were subjected to sequencing of the 16S rRNA gene and comparison with the M4-derived symbiont sequence, it turned out that (i) most of the colony-derived sequences exhibited 100% identity to the M4-derived symbiont sequence, indicating that the gut symbiotic bacteria of *M. gracilicorne* are generally cultivable, (ii) in several cases (e.g., two insects from Chikusei and one insect from Chiba), one or two non-symbiont sequences were detected, indicating that minor non-symbiont bacteria may often coexist with the major symbiotic bacteria in the M4 region of *M. gracilicorne*, and (iii) as for three insects from Takasaki, all of the eight sequences disagreed with the symbiont sequence and instead represented a variety of other bacterial sequences, suggesting the possibility that the gut symbiotic bacteria of *M. gracilicorne* of the Takasaki population might be exceptionally resistant to cultivation, although other possibilities, such as accidental sampling of unhealthy insects at Takasaki, should also be taken into account (Table 1; see Supplementary Table S2). In this context, it may be notable that a widely used herbicide, glyphosate, suppresses insect gut bacteria (Motta et al., 2018).

Transmission mode of gut symbiotic bacteria of *M. gracilicorne*

In this study, we developed a laboratory rearing system for *M. gracilicorne* supplied with fresh cucumbers (Fig. 1B). In our preliminary rearing trial in 2013, field-collected adult insects were kept in a rearing cage, they laid many eggs,

Table 1. Direct bacterial counts, colony formation units (CFUs), and symbiont detection rates among cultured colonies from dissected midgut M4 region of *M. gracilicorne*. For details, see Supplementary Table S2.

Sample ID[a]	Direct count per M4[b]	CFU per M4 on LB plate[b]	Symbiont detection rate among cultured colonies[b,c]
CKSI20_01	7.0×10^7	6.4×10^4	75.0% (6/8)
CKSI20_02	8.9×10^7	4.0×10^2	87.5% (7/8)
CKSI20_03	1.4×10^8	1.8×10^4	100% (8/8)
CHBA20_01	4.4×10^7	1.6×10^5	100% (8/8)
CHBA20_02	5.2×10^7	3.7×10^5	87.5% (7/8)
CHBA20_03	4.0×10^7	2.4×10^5	100% (8/8)
FKOK20_01	1.2×10^8	3.8×10^5	100% (8/8)
FKOK20_02	7.4×10^7	5.2×10^4	100% (8/8)
FKOK20_03	1.2×10^8	1.4×10^5	100% (8/8)
TKSK20_01	7.7×10^7	3.6×10^2	0.0% (0/8)
TKSK20_02	4.3×10^7	2.3×10^3	0.0% (0/8)
TKSK20_03	1.1×10^8	8.8×10^3	0.0% (0/8)

[a]Sample ID after Supplementary Table S1.
[b]Each field-collected mother was subjected to dissection and isolation of midgut M4 region. The isolated symbiotic organ was homogenized. One half of the homogenate was subjected to DNA extraction, PCR amplification, cloning and sequencing of bacterial 16S rRNA gene dominating the M4 symbiotic region. The remaining half of the homogenate was subjected to direct bacterial cell counting, and also serially diluted and spread onto LB agar plates for CFU counting. From the plates, eight single colonies were picked and subjected to DNA extraction, PCR amplification and direct sequencing of bacterial 16S rRNA gene.
[c]Percentage in terms of (number of symbiont-derived colonies/number of all colonies subjected to sequencing of bacterial 16S rRNA gene). A colony was regarded as symbiont-derived when the colony-derived sequence exhibited 100% match to the M4-derived sequence.

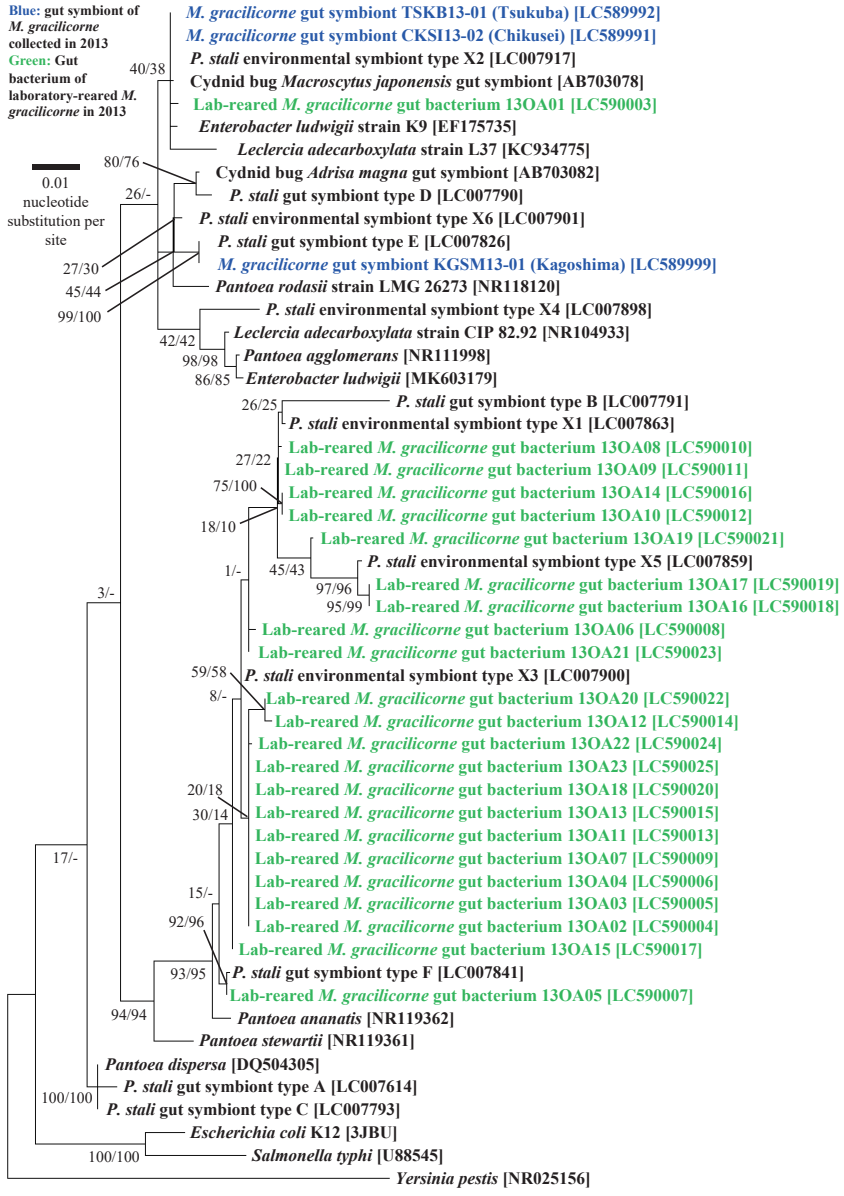

Fig. 3. Molecular phylogenetic placement of the gut bacteria of laboratory-reared adult insects of *M. gracilicorne*. Maximum-likelihood phylogeny inferred from 1332 aligned nucleotide sites of the 16S rRNA gene under the GTR+G+I model is shown, while neighbor-joining phylogeny exhibits substantially the same tree topology. Bootstrap probabilities are indicated at the nodes in the order of maximum-likelihood/neighbor-joining. Accession numbers for the sequences are shown in brackets. As for colors, see the notes on the upper left.

and some nymphs from the eggs attained adulthood, of which 23 adult insects were inspected for the bacterial 16S rRNA gene sequence from their dissected M4 region. Considering that stinkbugs of the superfamily Pentatomoidea generally transmit their gut symbiont vertically via egg surface contamination or deposition of symbiont-containing materials with eggs (Salem et al., 2015; Hosokawa and Fukatsu, 2020), we expected that the laboratory-reared adult insects of *M. gracilicorne* should inherit the gut symbiont maternally. Unexpectedly, however, most of the insects did not harbor the maternal gut symbiont: only one insect hosted the type X2 gut symbiont, and the remaining 22 insects were associated with a variety of *Pantoea*-allied γ-proteobacteria, the majority of which were allied to either the type X1, X3 or X5 potential symbiont of *P. stali* (Hosokawa et al., 2016a) (Fig. 3). These observations suggest the possibility that *M. gracilicorne* exhibits neither egg surface contamination nor vertical symbiont transmission. In 2020, in order to confirm this, we individually reared seven field-collected adult females, collected their eggs and nymphs serially, and PCR-amplified, cloned and sequenced partial bacterial 16S rRNA gene sequences from these samples (Table 2; see Supplementary Table S3). In the majority of the egg samples (8/14), none of eight clones per sample represented the symbiont sequence (Table 2), indicating the absence of stable smearing of the symbiotic bacteria onto eggs. Concordantly, in the majority of the nymphal samples (11/14), none or a few (0, 1 or 2) of eight clones per sample represented the symbiont sequence (Table 2), indicating the absence of stable vertical transmission of the symbiotic bacteria. Meanwhile, some eggs and nymphs exhibited the symbiont sequence at low frequencies (mostly one, two or three, and at most five, per eight clones) (Table 2), suggesting that the eggs and the nymphs may be occasionally contaminated by the symbiotic bacteria of either parental or environmental origins. The nymphs exhibited relatively higher symbiont infection frequencies than the eggs (Table 2), which is probably due to active symbiont exploitation by the nymphs. The non-symbiont 16S rRNA gene sequences obtained from the eggs and the nymphs were phylogenetically diverse (see Supplementary Table S3), which plausibly reflected the environmental microbiota. Notably, an *Ehrlichia*-like 16S rRNA gene sequence was frequently detected from the eggs and the nymphs (see Supplementary Table S3), which may represent a rickettsial facultative endosymbiont of *M. gracilicorne* as in *Nysius* seed bugs (Matsuura et al., 2012), and its microbial identity should be investigated in future studies.

Presumable benefit of gut symbiotic bacteria for survival of *M. gracilicorne*

Finally, we evaluated the biological consequences of the gut symbiotic bacteria in *M. gracilicorne*. We generated several experimental groups consisting of 100–400 eggs laid by field-collected adult females. In the symbiont-supplemented groups, first instar nymphs were provided with fresh cucumbers and symbiont-supplemented water, whereas in the control groups, first instar nymphs were supplied with fresh

Table 2. Vertical transmission rate of gut symbiotic bacteria from mother to offspring of *M. gracilicorne*. For details, see Supplementary Table S3.

Mother[a,b]	Egg1[b]	Nymph1[b]	Egg2[b]	Nymph2[b]
CKSI20-12	12.5% (1/8 clones)	12.5% (1/8 clones)	0.0% (0/8 clones)	12.5% (1/8 clones)
CKSI20-13	0.0% (0/8 clones)	12.5% (1/8 clones)	0.0% (0/8 clones)	0.0% (0/8 clones)
CKSI20-14	12.5% (1/8 clones)	25.0% (2/8 clones)	0.0% (0/8 clones)	37.5% (3/8 clones)
CKSI20-15	0.0% (0/8 clones)	0.0% (0/8 clones)	12.5% (1/8 clones)	62.5% (5/8 clones)
CKSI20-16	0.0% (0/8 clones)	37.5% (3/8 clones)	50.0% (4/8 clones)	25.0% (2/8 clones)
CKSI20-17	0.0% (0/8 clones)	0.0% (0/8 clones)	0.0% (0/8 clones)	12.5% (1/8 clones)
CKSI20-18	25.0% (2/8 clones)	25.0% (2/8 clones)	12.5% (1/8 clones)	25.0% (2/8 clones)

[a]Sample ID after Supplementary Table S1.
[b]Each field-collected mother was individually reared, and first-laid eggs and second-laid eggs were harvested. Two eggs from the first clutch were defined as "Egg1", and subjected to DNA extraction, PCR and cloning of bacterial 16S rRNA gene. For each of the egg samples, eight 16S rRNA gene clones were picked and sequenced. The remaining eggs were maintained, and a newborn nymph was sampled as "Nymph1", from which eight 16S rRNA gene clones were picked and sequenced in the same way. Similarly, two eggs and a nymph from the second clutch were defined as "Egg2" and "Nymph2", from which eight 16S rRNA gene clones were picked and sequenced in the same way.

cucumbers and sterilized water. From the second instar and on, the insects of both groups were provided with fresh cucumbers and sterilized water only. In 2019, we generated three symbiont-supplemented groups and three control groups. In all of the groups, while first instar nymphs suffered high mortality, second instar and older nymphs exhibited relatively good survivorship. The adult emergence rate, 25.6% (161/629), in the symbiont-supplemented groups was significantly higher than the adult emergence rate, 9.5% (52/548), in the control groups (Fig. 4A). In 2020, we repeated the rearing experiments with two symbiont-supplemented groups and two control groups, which yielded similar results: high mortality at the first instar and stable survivorship at the later stages, and significantly higher adult emergence rate in the symbiont-supplemented groups, 13.1% (62/474), than in the control groups, 4.7% (25/532) (Fig. 4B). These results suggested that the gut symbiont of *M. gracilicorne* may affect host's survival positively.

Origin of gut symbiotic bacteria in non-inoculated control insects of *M. gracilicorne*?

However, when we inspected the gut bacteria of the symbiont-supplemented adult insects and the control adult insects obtained in 2020, perplexing patterns of bacterial infections emerged. As expected, all of the symbiont-supplemented insects were infected with the gut symbiotic bacteria of the inoculated type (Table 3). On the other hand, unexpectedly, some of the control insects (five of eight) were also infected with the gut symbiotic bacteria of the inoculated type, whereas the other control insects (three of eight) were associated with bacteria allied to cultivable gut symbionts (e.g., type F, type X5, etc.) of *P. stali* and other stinkbug species (Table 3; see Supplementary Figure S2). How could the gut symbiotic bacteria be acquired by the control insects despite the absence of symbiont inoculation? There are two possible routes: (i) experimental contamination from the symbiont-supplemented insect populations maintained in parallel, and (ii) environmental contamination from fresh cucumbers supplied as food. The rearing experiments of *M. gracilicorne* from egg to adult continued for as long as three months, during which accidental contamination of the symbiotic bacteria from the symbiont-supplemented cages to the control cages might have occurred despite our careful experimental handling of them. Alternatively, considering that surface sterilization of cucumbers may facilitate rotting and cause negative effects on the insect rearing, we used fresh cucumbers washed with tap water as laboratory food for *M. gracilicorne*, which can be the source of environmental *Pantoea*-allied bacteria capable of symbiosis with stinkbugs (Hosokawa et al., 2016a). Notably, some 16S rRNA gene sequences detected in the control insects were nearly, but not completely, identical to the sequence of the inoculated symbiont (ex. 20OA–Sym03, 20OA–Sym04 and 20OA–Sym07 in Table 3), which favors the idea of food-derived bacterial infections. It is also notable that all of the non-inoculated control adult insects we inspected were not aposymbiotic but infected with *Pantoea*-allied bacteria (Table 3; see Supplementary Figure S2). In this context, it seems relevant that, even when nymphs of the stinkbug *P. stali* were maintained under aseptic conditions, accidental bacterial infections occasionally occurred from food peanuts, and the insects infected with the non-symbiont bacteria exhibited improved growth and survival in comparison with the bacteria-free insects (Nishide et al., 2017). Considering all of these results, observations and circumstances taken together, our current hypothesis to account for the survival patterns of the symbiont-inoculated insects and the control insects (Fig. 4) is that (i) *M. gracilicorne* requires *Pantoea*-allied gut symbiotic bacteria for normal growth and survival, (ii) the symbiont-supplemented nymphs acquired the symbiotic bacteria immediately, (iii) by contrast, the control nymphs acquired the food-derived *Pantoea*-allied bacteria only gradually and sporadically, and (iv) consequently, the symbiont-supplemented insects exhibited better survival than the control insects. Needless to say, we recognize that there are a number of uncertainties regarding this scenario, including infection dynamics, phenotypic effects, and presence on cucumbers of the *Pantoea*-allied gut bacteria, which will be investigated in future studies.

Conclusion and perspective

In conclusion, the gut bacterial symbiosis in *M. gracilicorne* is unique among pentatomoid stinkbugs: the microbial nature of the symbionts as *Pantoea*-allied γ-proteobacteria and the symbiont localization within the midgut crypts are typical of pentatomoid stinkbugs, whereas the lack of vertical symbiont transmission and the environmental symbiont acquisition are exceptional among pentatomoid stinkbugs, being rather close to the β-proteobacterial gut symbiosis found among coreoid and lygaeoid stinkbugs. The unique gut symbiosis of *M. gracilicorne* may provide unique opportunities to better understand the ecology and

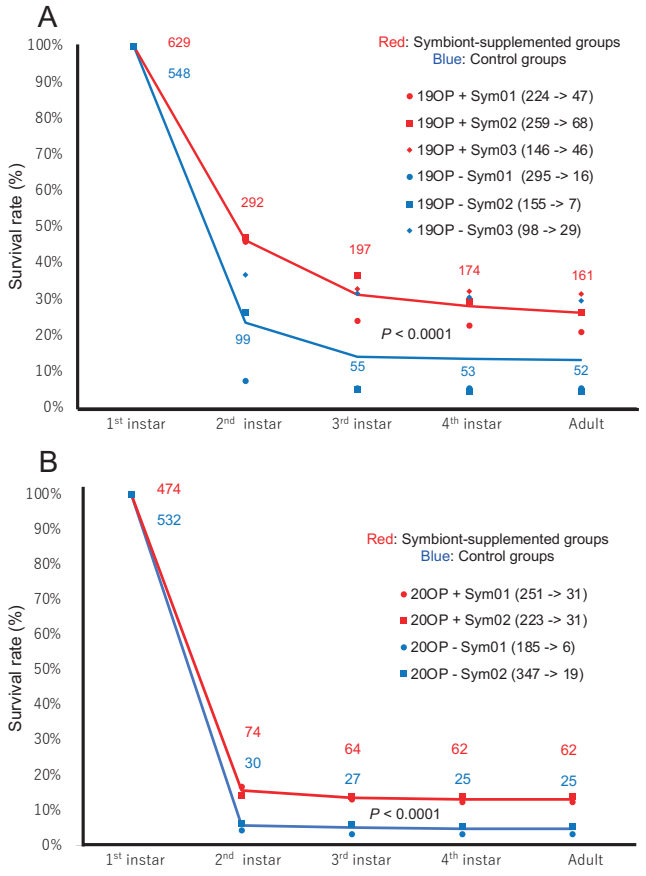

Fig. 4. Survival curves of laboratory-reared *M. gracilicorne* with or without supplementation of cultured symbiotic bacteria. **(A)** The result of the experiment in 2019. **(B)** The result of the experiment in 2020. Red symbols and blue symbols show survival rates of the symbiont-supplemented groups and the control groups, respectively. Red lines and blue lines are drawn based on the mean survival rates of the symbiont-supplemented groups and the control groups, respectively, with numbers indicating the total numbers of surviving insects for each of the treatment groups. The initial number of first instar nymphs and the final number of adult insects for each treatment group are shown on the upper right. The survival rates were significantly different between the symbiont-supplemented groups and the control groups in both the 2019 and 2020 experiments ($P < 0.0001$, generalized Wilcoxon test). The high mortality in the first instar is probably because the cucumber peel is too thick for tiny first instar nymphs to penetrate with the mouthpart.

evolution of symbiosis, given that whether symbiont acquisition occurs vertically or environmentally pivotally affects the evolutionary fate of host-symbiont relationships such as virulence, infection cost, infection stability, fitness effects, symbiont genome reduction, host-symbiont interdependence, etc. (Ewald, 1987; Kikuchi et al., 2007; Bright and Bulgheresi, 2010; McCutcheon and Moran, 2012; Russell, 2019). Although this study is the first to unequivocally report the cultivable and environmentally acquired gut symbiotic γ-proteobacteria in the Pentatomoidea, we suspect that many other cases are to be found and characterized. For example, see Fig. 2, in which the gut symbiotic bacteria of the soil-dwelling cydnid stinkbugs *Macroscytus japonensis* and *Adrisa magna* (see Hosokawa et al., 2012a) are genetically almost identical to the cultivable gut symbiotic bacteria

Table 3. Bacterial infections in laboratory-reared *M. gracilicorne* with and without supplementation of cultured symbiotic bacteria.

Sample ID[a]	Symbiont detection rate[b,c]	Consensus sequence identity to inoculated symbiont sequence[d,e]
From experimental groups with administration of cultured symbiotic bacteria (20OP+Sym01 or 20OP+Sym02)		
20OA+Sym01	100% (7/7 clones)	100% (515/515) [LC594073]
20OA+Sym02	87.5% (7/8 clones)	100% (515/515) [LC594074]
20OA+Sym03	75.0% (6/8 clones)	100% (515/515) [LC594075]
20OA+Sym04	100% (8/8 clones)	100% (515/515) [LC594076]
20OA+Sym05	100% (8/8 clones)	100% (515/515) [LC594077]
20OA+Sym06	87.5% (7/8 clones)	100% (515/515) [LC594078]
20OA+Sym07	75.0% (6/8 clones)	100% (515/515) [LC594079]
20OA+Sym08	50.0% (4/8 clones)	100% (515/515) [LC594080]
From control groups without administration of cultured symbiotic bacteria (20OP–Sym01 or 20OP–Sym02)		
20OA–Sym01	0.0% (0/8 clones)	98.8% (509/515) [LC594081]
20OA–Sym02	0.0% (0/8 clones)	98.1% (505/515) [LC594082]
20OA–Sym03	100% (6/6 clones)	99.8% (514/515) [LC594083]
20OA–Sym04	87.5% (7/8 clones)	99.8% (514/515) [LC594084]
20OA–Sym05	75.0% (6/8 clones)	100% (515/515) [LC594085]
20OA–Sym06	66.7% (4/6 clones)	100% (515/515) [LC594086]
20OA–Sym07	100% (8/8 clones)	99.8% (514/515) [LC594087]
20OA–Sym08	0.0% (0/8 clones)	97.7% (503/515) [LC594088]

[a]See Supplementary Table S1.
[b]In the symbiont-supplemented groups 20OP+Sym01 and 20OP+Sym02, first instar nymphs were reared with fresh cucumbers and water supplemented with cultured symbiotic bacteria (originally derived from the strain CKSI13-01), they were transferred to new rearing containers without symbiont supplementation upon second instar molt and on, and after adult emergence, eight adult insects (20OA+Sym01–20OA+Sym08) were randomly selected and individually subjected to dissection of midgut M4 region. The dissected symbiotic organ was subjected to DNA extraction, PCR and cloning of bacterial 16S rRNA gene. For each of the adult insects, eight 16S rRNA gene clones were picked and sequenced. In the control mass-reared experimental groups 20OP–Sym01 and 20OP–Sym02, the same experimental procedures were conducted except that first instar nymphs were provided with sterilized water.
[c]A colony was regarded as symbiont-derived when the colony-derived sequence exhibited > 99% match to the CKSI13-01-derived sequence.
[d]For each insect, a consensus sequence of the 16S rRNA sequences was generated, and compared with the CKSI13-01-derived sequence.
[e]Percentage in terms of (number of matched nucleotide sites/number of aligned nucleotide sites), where gap-containing sites were omitted from the alignment, [accession number].

of *M. gracilicorne*; the cultivable gut symbiotic bacteria types C, D, E and X2 of the pentatomid stinkbug *P. stali* (see Hosokawa et al., 2016a), are phylogenetically intermingled with the cultivable gut symbiotic bacteria of *M. gracilicorne*; and the cultivable gut symbiotic bacteria types C, D and E are also found in subtropical stinkbug species such as *Axiagastus rosmarus* (Pentatomidae), *Lampromicra miyakona* (Scutelleridae) and *Solenosthedium chinense* (Scutelleridae) (see Hosokawa et al., 2016a). Therefore, by

comparatively investigating the vertically transmitted and environmentally acquired gut symbiotic associations among diverse pentatomoid stinkbugs, we will be able to empirically test a variety of evolutionary hypotheses regarding symbiosis, which we believe should be a promising direction of future studies.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

TN, TH and TF designed the study. TH performed most of the research works in 2013. TN conducted most of the research works in 2019 and 2020. XYM and RK performed TEM and FISH, respectively. MM managed the progress of the study and was involved in the data analysis. TN and TF wrote the paper. All authors approved the final manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: ZS-O-2020-0163.R1)

Supplementary Figure S1. Collection localities of the samples of *M. gracilicorne* used in this study.

Supplementary Figure S2. Molecular phylogenetic placement of the gut bacteria of laboratory-reared *M. gracilicorne* with or without supplementation of cultured symbiotic bacteria.

Supplementary Table S1. Samples of *M. gracilicorne* used in this study.

Supplementary Table S2. Direct sequencing of bacterial 16S rRNA gene sequences from cultured bacterial colonies on LB plates derived from crushed midgut M4 region of *M. gracilicorne*.

Supplementary Table S3. Cloning and sequencing of bacterial 16S rRNA gene sequences from maternal symbiotic M4 region, and offspring eggs and newborn nymphs of *M. gracilicorne.*

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