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Bacteriocytes and *Blattabacterium* Endosymbionts of the German Cockroach *Blattella germanica*, the Forest Cockroach *Blattella nipponica*, and Other Cockroach Species

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Cockroaches are commonly found in human residences and notorious as hygienic and nuisance pests. Notably, however, no more than 30 cockroach species are regarded as pests, while the majority of 4,500 cockroaches in the world are living in forest environments with little relevance to human life. Why some cockroaches have exceptionally adapted to anthropic environments and established pest status is of interest. Here we investigated the German cockroach *Blattella germanica*, which is a cosmopolitan pest species, and the forest cockroach *Blattella nipponica*, which is a wild species closely related to *B. germanica*. In contrast to easy rearing of *B. germanica*, laboratory rearing of *B. nipponica* was challenging—several trials enabled us to keep the insects for up to three months. We particularly focused on the distribution patterns of specialized cells, bacteriocytes, for harboring endosymbiotic *Blattabacterium*, which has been suggested to contribute to host's nitrogen metabolism and recycling, during the postembryonic development of the insects. The bacteriocytes were consistently localized to visceral fat bodies filling the abdominal body cavity, where a number of single bacteriocytes were scattered among the adipocytes, throughout the developmental stages in both females and males. The distribution patterns of the bacteriocytes were quite similar between *B. germanica* and *B. nipponica*, and also among other diverse cockroach species, plausibly reflecting the highly conserved cockroach-*Blattabacterium* symbiotic association over evolutionary time. Our study lays a foundation to experimentally investigate the origin and the processes of urban pest evolution, on account of possible involvement of microbial associates.

Key words: cockroach, *Blattella*, symbiont, *Blattabacterium*, bacteriocyte, symbiosis, pest, evolution

INTRODUCTION

Cockroaches (Insecta: Blattodea) embrace over 4500 species in the world, of which most prosper in tropical/subtropical regions, while some thrive in temperate regions (Grimaldi and Engel, 2005; Bell et al., 2007). Some species, such as German, American, Australian, oriental, and smokybrown cockroaches, are commonly found in human residences, where they infest and contaminate food stuffs and vector pathogenic microbes, and are thereby regarded as notorious hygienic and nuisance pests. Actually, however, such so-called "pest" cockroaches constitute no more than 30 species in the world, and the majority of the cockroaches reside in forest environments with little relevance to human life (Schal and Hamilton, 1990; Bell et al., 2007). Why and how these specific cockroaches have exceptionally adapted to anthropic environments and established pest status is of both basic and applied interest.

Many insects are associated with bacterial endosymbionts, which usually play important biological roles for the host insects (Buchner, 1965; Bourtzis and Miller, 2003). Historically, the cockroach was among the first insects recognized as having intracellular bacteria-like particles, which were highlighted in the dawn of the conceptualization of endosymbiosis. It was in the late 19th century that Blochmann (1887) discovered discrete cells full of rod-shaped objects, which he called bacteroids, within the abdominal fat body of the German cockroach *Blattella germanica* and the oriental cockroach *Blatta orientalis*. Since then, for decades, such

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intracellular particles have been termed "Blochmann bodies", being the subject of recurrent controversies as to whether the bacteria-like particles are actually microorganisms or endogenous intracellular structures (Buchner, 1965; Lanham, 1968). Later the bacterial symbiont conserved among diverse cockroaches was designated as *Blattabacterium* (Hollande and Favre, 1931), and molecular phylogenetic analysis identified it as a member of the Bacteroidetes (Bandi et al., 1994).

Probably because of the early discovery of the endosymbiosis and the easy accessibility and the experimental tractability of the home-dwelling pest, in the early to mid 20th century, the cockroach-*Blattabacterium* association represented the best-studied insect-microbe symbiotic system, yielding a considerable body of research reports. There are many histological descriptions of the symbiotic bacteria and the bacteriocytes, ranging from classic hand-drawn sketches to more recent light and electron microscopic observations (Blochmann, 1887; Wheeler, 1889; Gier, 1936; Koch, 1949; Bush and Chapman, 1961; Walker, 1965; Milburn, 1966; Cochran et al., 1979; Sacchi et al., 1993, 1996, 1998a, b, 2000; Laudani et al., 1995; Lambiase et al., 1997; Park et al., 2013). The vertical transmission processes of the symbiotic bacteria and formation processes of the bacteriocytes were histologically described in detail, particularly focusing on the processes during embryogenesis (Gier, 1936; Koch, 1949; Sacchi et al., 1996, 2000; Lambiase et al., 1997). Many researches attempted to cultivate the symbiotic bacteria *in vitro*, but, despite an array of erroneous reports of success (e.g., Mercier, 1907; Gropengiesser, 1925; Glaser, 1930; Hoover, 1945; Pierre, 1964), the attempts finally turned out to be in vain (Gier, 1947; Brooks and Richards, 1966). Biochemical, physiological and functional works on the symbiotic relationship were conducted, particularly focusing on possible involvement in nitrogen metabolism and recycling, on the grounds that (i) cockroaches excrete not uric acid but ammonia, (ii) cockroaches retain crystalized uric acid within special cells called urocytes within the abdominal fat body, (iii) the uric acid crystals accumulate in fully fed insects and disappear in starved insects, and (iv) the urocytes are closely associated with the bacteriocytes in the fat body (Walker, 1965; Mullins and Cochran, 1972, 1974, 1976; Cochran et al., 1979; Cochran, 1985; Wren and Cochran, 1987; Sacchi et al., 1993; Park et al., 2013). However, despite recent numerous molecular phylogenetic works (Bandi et al., 1994, 1995; Lo et al., 2003; Clark et al., 2001; Clark and Kambhampati, 2003; Maekawa et al., 2005a, b) and completed symbiont genomes (Sabree et al., 2009; López-Sánchez et al., 2009; Neef et al., 2011; Huang et al., 2012; Sabree et al., 2012; Tokuda et al., 2013; Patino-Navarrete et al., 2014), biological and functional aspects of the cockroach-*Blattabacterium* symbiosis are still elusive.

Recently, we started working on the German cockroach *B. germanica* (Fig. 1A) and the forest cockroach *B. nipponica* (Fig. 1B), because (i) *B. germanica* is a cosmopolitan pest species, easily maintainable in the laboratory, and established as a model cockroach species (Rust et al., 1995), (ii) *B. nipponica* is very closely related to *B. germanica* taxonomically and morphologically (Fig. 1A–D) (Asahina, 1963, 1991), (iii) notwithstanding this, while *B. germanica* is a pest species found indoors only, *B. nipponica* is a wild species living in natural forests (Asahina, 1991; Harunari et al., 2007), (iv) therefore, comparative studies on these species would provide insight into what traits, adaptation and evolution underlie the pest status of the cockroaches, and (v) considering the presumably nutrition-rich lifestyle of *B. germanica* in contrast to the nutrition-limited lifestyle of *B. nipponica*, some differences may be found in the functional aspects of the endosymbiosis between the pest and non-pest species.

In this study, we report (i) distribution patterns of bacteriocytes and *Blattabacterium* during the postembryonic development of *B. germanica*, (ii) trials of laboratory rearing of *B. nipponica*, (iii) comparison of distribution patterns of bacteriocytes and *Blattabacterium* between *B. germanica* and *B. nipponica*, and (iv) inspection of distribution patterns of bacteriocytes and *Blattabacterium* among diverse cockroach species.

MATERIALS AND METHODS

Insect materials

A stock population of the German cockroach *B. germanica*, which was derived from around 40 individuals provided by Ikari Shodoku Co., Ltd., was established at the National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan. The insects were reared in plastic containers at 27°C under a 12 h light and 12 h dark regime in an incubator (MLR-352H-PJ, Panasonic) with an insect feed (Insect Diet I, Oriental Yeast Co., Ltd.) and water. Adult females carrying an ootheca (a large egg case containing 30–40 eggs) were isolated individually into small plastic containers (9 cm × 9 cm × 14 cm), since the ootheca cannot be separated from the mother. Once the eggs hatched, the females were returned to the stock population while the nymphs were kept in the containers. Daily monitoring of the nymphs ensured accurate instar staging. Sexing of the insects was performed as described (Ross and Cochran, 1960).

The forest cockroach *B. nipponica*, the Japanese cockroach *Periplaneta japonica*, and the smokybrown cockroach *Periplaneta fuliginosa* were collected by setting pitfall traps in the AIST campus, Tsukuba, Japan, using dog food as bait and Vaseline to prevent escape. We also collected *B. nipponica* from fallen leaves accumulated on the forest floor using insect nets, and attempted to maintain the insects in the laboratory using the rearing method for *B. germanica* with modifications, as described later.

Blatta lateralis (also known under the synonym *Shelfordella lateralis*) and *Blaptica dubia* were purchased at pet stores, and were reared as described above for *B. germanica*.

DNA analysis

After surface sterilization in 70% ethanol, the insects were subjected to dissection and isolation of fat bodies in phosphate buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4; 0.02% KH_2PO_4 [pH 7.4]), and then DNA extraction using a QIAmp DNA Mini Kit (Qiagen). A 1.5 kb region of the bacterial 16S rRNA gene was amplified from the DNA samples by PCR using Tks-Gflex DNA polymerase (Takara) with the primers 10FF (5′-AGT TTG ATC ATG GCT CAG GAT-3′) and 1515R (5′-GTA CGG CTA CCT TGT TAC GAC TTA G-3′) (Moran et al., 2005) under a temperature profile of 98°C for 1 min followed by 35 cycles of 98°C for 10 sec, 55°C for 15 sec and 68°C for 90 sec, and a final incubation at 68°C for 10 min. We also used the following primers 16SA2 (5′-GTG CCA GCA GCC GCG GTA ATA C-3′), 16SB2 (5′-CGA GCT GAC GAC ARC CAT GCA-3′) (Fukatsu and Nikoh, 1998), and EUB338 (5′-GCT GCC TCC CGT AGG AGT-3′) (Amann et al., 1990) for DNA sequencing. The PCR products were checked by electrophoresis in agarose gels and staining with ethidium bromide, and subjected to DNA sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130xl Genetic Analyzer (Applied Bio-

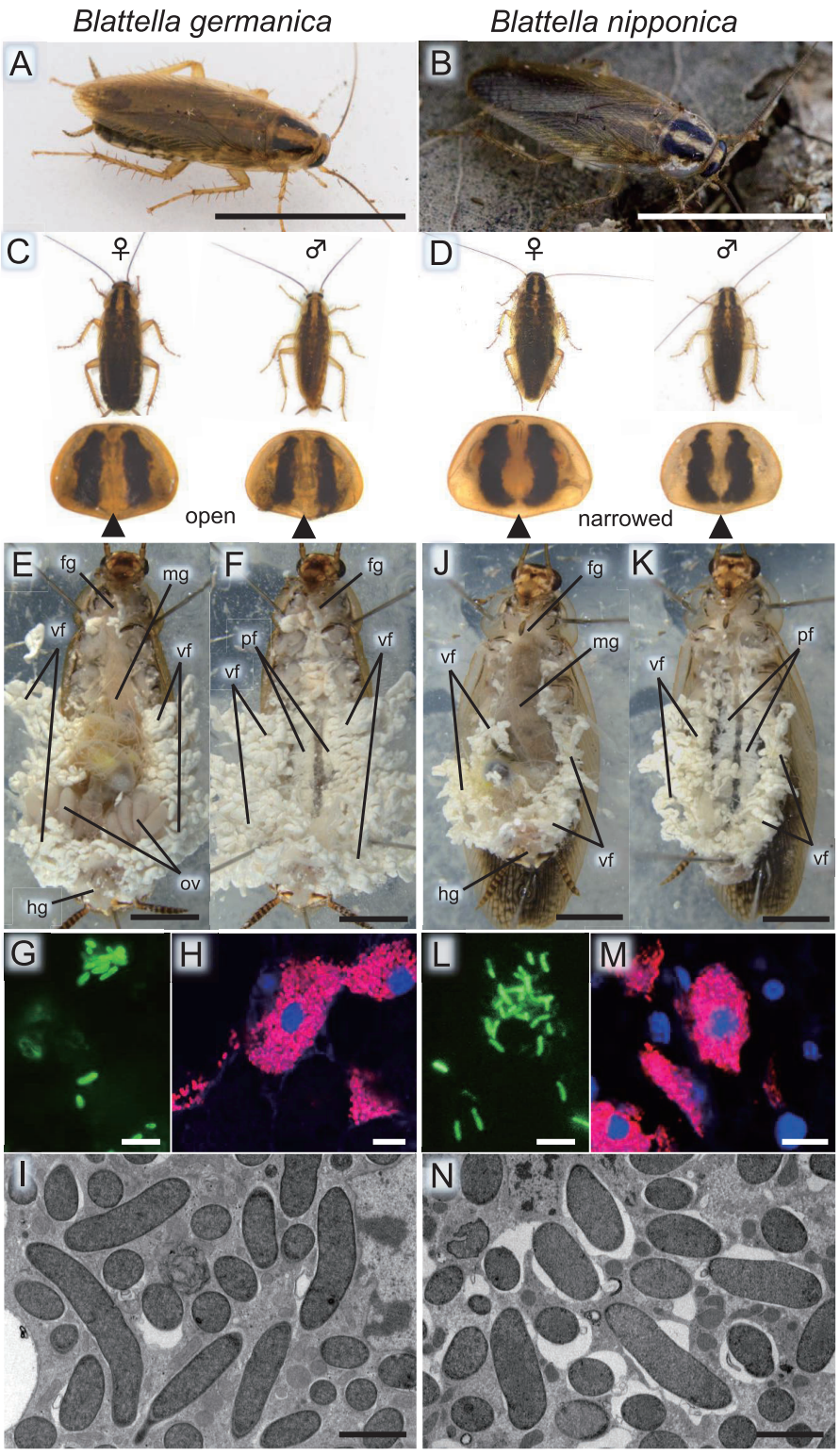

Fig. 1. Morphology, anatomy, fat bodies, bacteriocytes, and *Blattabacterium* symbionts of *Blattella* cockroaches. **(A, C, E–I)** *B. germanica*. **(B, D, J–N)** *B. nipponica*. **(A, B)** Adult insects. **(C, D)** Morphological features of adult females and males. **(E, F, J, and K)** Dissected adult insects. Visceral fat bodies and other internal organs are shown in **(E)** and **(J)**, whereas peripheral fat bodies on the ventral plates were exposed by removing the organs in **(F)** and **(K)**. Note that laboratory-reared *B. germanica* possesses highly developed fat bodies in comparison with field-collected *B. nipponica*. Abbreviations: fg, foregut; hg, hindgut; mg, midgut; ov, ovary; pf, peripheral fat body; vf, visceral fat body. **(G, L)** Fluorescence microscopic images of symbiont cells stained with SYTOX Green. **(H, M)** FISH images of bacteriocytes visualized using a fluorescent probe targeting symbiont's 16S rRNA. Red and blue signals show symbiont cells and host nuclear DNA, respectively. **(I, N)** TEM images of symbiont cells. Bars show 1 cm in **(A)** and **(B)**, 2.5 mm in **(E)**, **(F)**, **(J)** and **(K)**, 20 μm in **(G)**, **(H)**, **(L)** and **(M)**, and 2 μm in **(I)** and **(N)**.

systems). The bacterial 16S rRNA gene sequences were deposited in the DNA Data Bank of Japan under the accession numbers LC537904–LC537909.

Molecular phylogenetic analysis

Nucleotide sequences were multiple aligned using MAFFT v7.407 (Katoh and Standley, 2013) and poorly aligned regions were removed using trimAl v1.4 (Capella-Gutiérrez, 2009). Molecular phylogenetic analyses were conducted by neighbor-joining, maximum-likelihood, and Bayesian methods. Neighbor-joining phylogenies were constructed using MEGA ver. X (Kumar et al., 2018) with 1,000 bootstrap replicates. Maximum-likelihood phylogenies were constructed using RAxML v8.2.12 (Stamatakis, 2014) with 1000 bootstrap replicates. Bayesian phylogenies were inferred using MrBayes v3.2.6 (Ronquist et al., 2012). A general time reversible model with gamma distribution, which was selected by a model estimation program implemented in MEGA, was used for both the maximum-likelihood and Bayesian methods.

Direct symbiont observation

The insects were dissected in PBS, and the isolated pieces of visceral fat bodies were placed in a plastic tube with 60 μl of SYTOX Green solution diluted 1/30,000 in PBS. The tissues were lightly agitated using a pestle to break the tissue apart while ensuring that the bacterial cells would not be damaged. The suspension was incubated for 15 min at room temperature in darkness, dropped onto a glass slide, covered and crushed with a coverslip, and observed under an epifluorescence microscope (DFC 7000 T, Leica).

Histological processing

For whole insect sectioning, the insects were fixed in Carnoy's solution (ethanol: chloroform: acetic acid = 6: 3: 1) for 12 h or longer, and preserved in 100% ethanol in a refrigerator until use. Before histological preparation, either the lateral body edges or the head of the insects were incised by a razor blade to facilitate infiltration of reagents into the insect tissues. For dissected tissue sectioning, the insects were dissected in PBS, and the isolated fat bodies were fixed in Carnoy's solution for 12 h as well. Subsequently, the samples were incubated in PBT (PBS containing 0.1% Tween 20) overnight, and then washed with 1 M Tris buffer (pH 9.0) several times until the color of the samples became somewhat translucent, by which accumulated uric acid in the insect tissues was removed. The fat bodies of cockroaches accumulate crystalized uric acid especially when feeding on nitrogen-rich diets (Walker, 1965; Cochran et al., 1979), which may disturb tissue sectioning and fluorescence imaging. Then, the samples were washed, dehydrated and cleared through a water-ethanol-xylene series, embedded in paraffin, processed into serial tissue sections (10 μm thick) on a rotary microtome (RM2255, Leica), and mounted on glass slides.

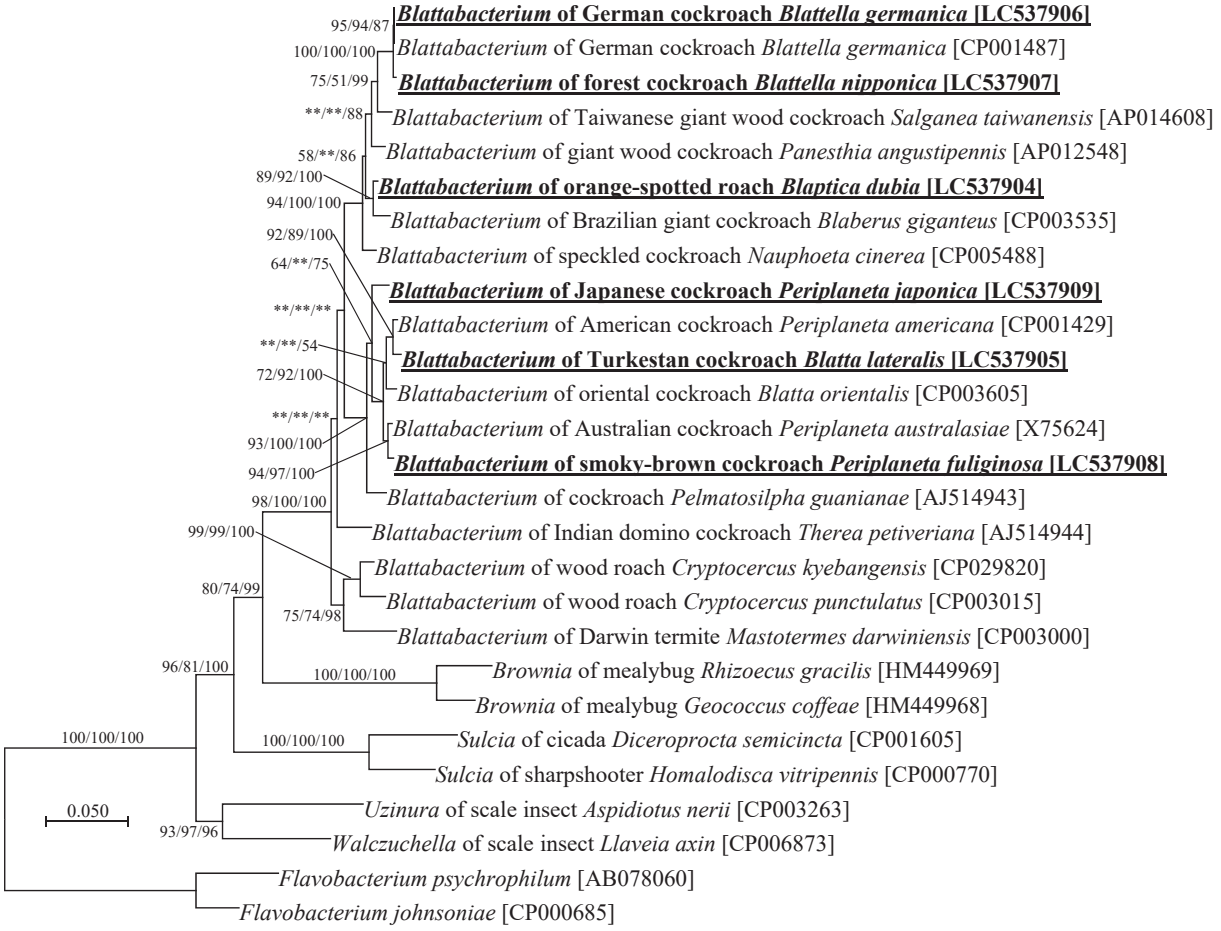

Fig. 2. Phylogenetic relationship of *Blattabacterium* symbionts of the cockroaches examined in this study on the basis of 16S rRNA gene sequences. A maximum-likelihood phylogeny inferred from 1246 aligned nucleotide sites is shown. Bootstrap probabilities of maximum-likelihood (ML) and neighbor-joining (NJ) analyses and posterior probabilities of Bayesian (BA) analysis are indicated at the nodes in the order of ML/NJ/BA. The symbiont sequences determined in this study are underlined. Nucleotide sequence accession numbers are indicated in brackets.

The tissue samples were deparaffinized and hydrated through a xylene-ethanol-water series, and subjected either to FISH or to conventional histological procedures including hematoxylin-eosin staining.

Fluorescence in situ hybridization (FISH) on tissue sections

FISH of tissue sections was conducted essentially as described previously (Koga et al., 2009; Koga et al., 2013). The tissue sections on the glass slides were overlayed with 150 μl of a hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% SDS, 30% formamide [w/v], 100 pmol/ml probe Sul664R [5′-Alexa555-CCM CAC ATT CCA GYT ACT CC-3′]), and then covered with coverslips, and incubated in a humidified container at room temperature for several hours. After washing with PBT, the samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI), mounted in 90% glycerol, and observed under an epifluorescence microscope (DFC 7000 T, Leica) or a laser scanning confocal microscope (LSM710, Zeiss).

Whole-mount FISH

Whole first instar nymphs or isolated fat bodies were subjected to whole-mount FISH. The samples were fixed in Carnoy's solution, and washed thoroughly with PBT and 1 M Tris buffer (pH 9.0) to reduce crystalized uric acid accumulated in the tissues. Then, the samples were treated with alcoholic hydrogen peroxide solution (6% H_2O_2 in 80% ethanol) for two weeks, during which the solution was replaced every two or three days, to reduce autofluorescence of the insect tissues (Koga et al., 2009). Whole-mount FISH was conducted essentially as described previously (Koga et al., 2013). After thorough washing in PBT, the samples were incubated in the hybridization buffer in plastic tubes at room temperature overnight on a lab shaker. After washing with PBT three times for 10 min each, the samples were counterstained with DAPI, thoroughly washed and placed on glass slides, mounted in 90% glycerol, and observed under a fluorescence dissection microscope (DFC 7000 T, Leica) and/or a laser scanning confocal microscope (LSM710, Zeiss). Digital images were taken, merged and adjusted using Affinity photo Image editing software version. 1.8 (Serif Europe).

Transmission electron microscopy (TEM)

The insects were dissected in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The isolated visceral fat bodies were prefixed with the fixative at 4°C overnight, postfixed with 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) at 4°C for 60 min, dehydrated through a water-ethanol series, embedded in Epon812 resin, processed into ultrathin sections (around 80 nm thick) on an ultramicrotome (EM UC7, Leica), mounted on copper meshes, stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (H-7600, Hitachi).

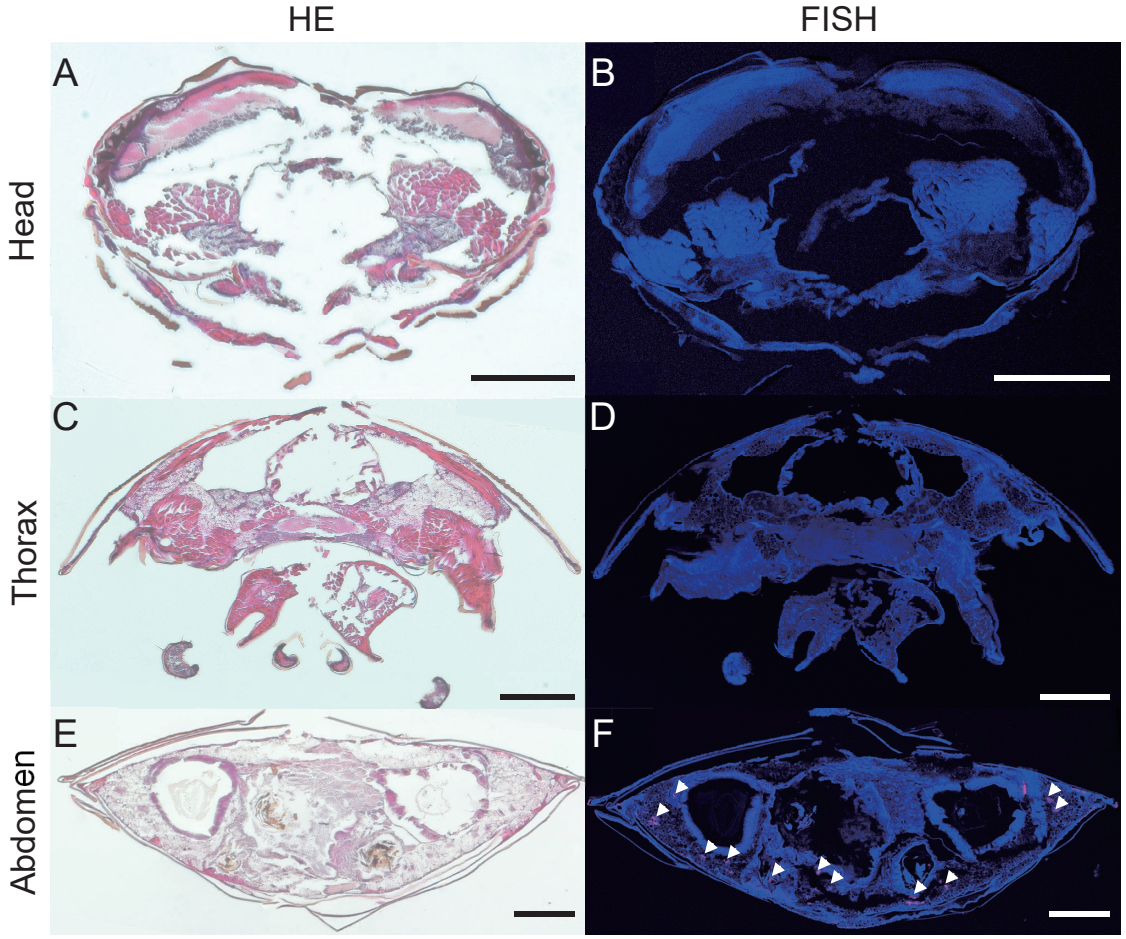

Fig. 3. Distribution of bacteriocytes in *B. germanica*. Cross sections of a first instar nymph at head **(A, B)**, thorax **(C, D)** and abdomen **(E, F)** are shown. Left column, light microscopic images of tissue sections stained with hematoxylin and eosin; right column, FISH images of tissue sections in which bacteriocytes are visualized in red (arrowheads) and host nuclear DNA is counterstained in blue. Bars show 200 μm.

RESULTS AND DISCUSSION

Detection of bacteriocytes and *Blattabacterium* in *B. germanica*

The body cavity of laboratory-reared *B. germanica* was full of well-developed fat bodies (Fig. 1E, F). Fluorescence microscopy, FISH and TEM detected rod-shaped symbiont cells and symbiont-harboring bacteriocytes within abdominal fat bodies (Fig. 1G–I). Molecular phylogenetic analysis based on the bacterial 16S rRNA gene sequence identified the fat body-inhabiting symbiotic bacteria as *Blattabacterium* (Fig. 2). Serial tissue sectioning of whole first instar nymphs and FISH detection of the symbiont revealed that the bacteriocytes are specifically detected in the abdominal fat bodies (Fig. 3). Within the abdomen of *B. germanica*, we identified two distinct types of fat bodies: visceral fat bodies filling the body cavity around the intestine (Figs. 1E, 4A) and peripheral fat bodies attached to the inner body wall (Figs. 1F, 4B). Tissue sectioning and FISH of dissected fat bodies demonstrated that the bacteriocytes are not found in the peripheral fat bodies but present only in the visceral fat bodies, where a number of single bacteriocytes are scattered among adipocytes (Fig. 4C–F).

Bacteriocytes and *Blattabacterium* during postembryonic development of *B. germanica*

After emerging from oothecae, first instar nymphs of *B. germanica* molt six times to become adults, which takes around six weeks under our rearing condition at 27°C. Throughout the postembryonic developmental course, female and male insects were processed into whole body tissue sections and subjected to FISH detection of the symbiont-harboring bacteriocytes. We observed that the distribution patterns of the bacteriocytes are substantially the same across all the developmental stages and irrespective of sex: single bacteriocytes are scattered among adipocytes within the visceral fat bodies in the abdomen (Fig. 5).

Collection and rearing of *B. nipponica*

The forest cockroach *B. nipponica* (Fig. 1B) is very close to the German cockroach *B. germanica* (Fig. 1A) not only taxonomically but also morphologically. At a glance,

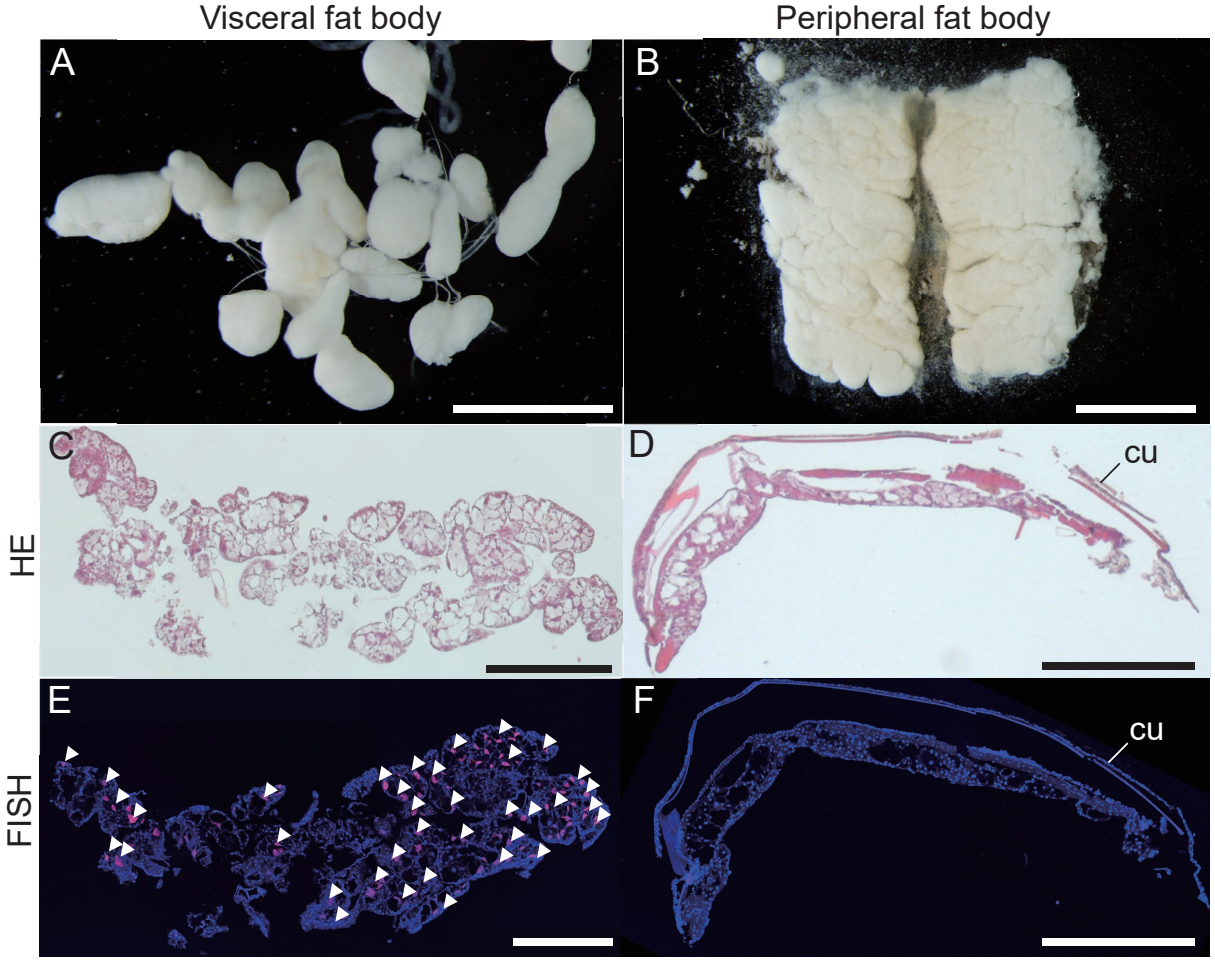

Fig. 4. Distribution of bacteriocytes in dissected fat bodies of *B. germanica*. **(A)** Visceral fat bodies and **(B)** peripheral fat bodies dissected from an adult insect. **(C, E)** Tissue sections of visceral fat bodies. **(D, F)** Tissue sections of peripheral fat bodies. **(C, D)** Light microscopic images of tissue sections stained with hematoxylin and eosin. **(E, F)** FISH images of tissue sections in which bacteriocytes are visualized in red (arrowheads) and host nuclear DNA is counterstained in blue. "cu" indicates cuticle of ventral plate. Bars show 1 mm in **(A)** and **(B)**, and 500 μm in **(C)**–**(F)**.

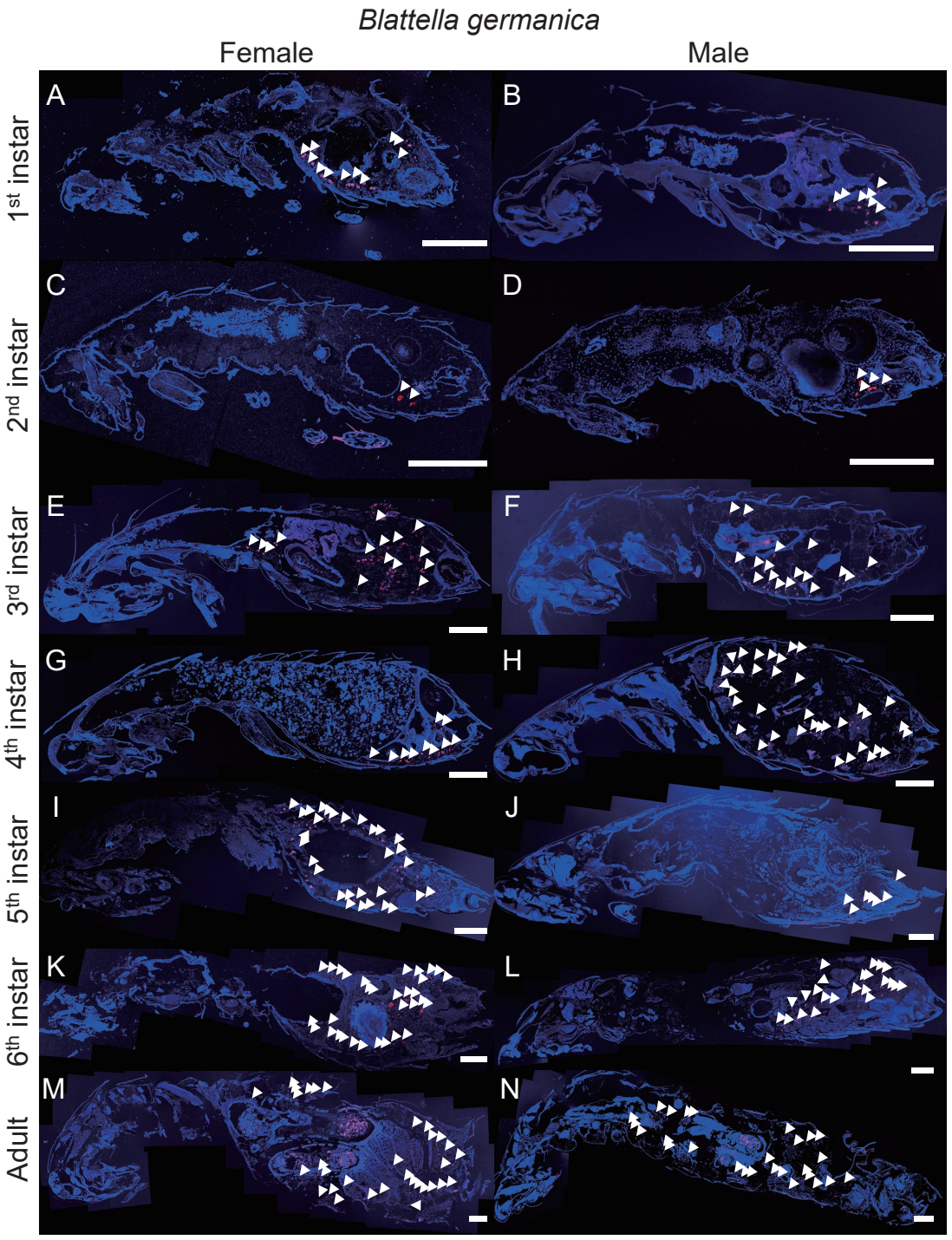

Fig. 5. Distribution of bacteriocytes throughout the postembryonic development of *B. germanica*. **(A, B)** First instar nymphs. **(C, D)** Second instar nymphs. **(E, F)** Third instar nymphs. **(G, H)** Fourth instar nymphs. **(I, J)** Fifth instar nymphs. **(K, L)** Sixth instar nymphs. **(M, N)** Adults. Females and males are shown on left and right, respectively. FISH images of sagittal whole body sections are displayed (left, head; right, abdomen; top, dorsal; bottom, ventral), in which bacteriocytes are visualized in red (arrowheads) and host nuclear DNA is counterstained in blue. Bars show 500 μm.

they look very similar to each other, but *B. nipponica* is characterized by longer wings and narrowed posterior ends of paired black stripes on the pronotum in comparison with *B. germanica* (Fig. 1C, D) (Asahina, 1963, 1991). Behaviorally, *B. germanica* seldom flies but *B. nipponica* actively flies (Asahina, 1991). Ecologically, *B. germanica* mainly lives in human residences and continuously reproduces there, being notorious as a hygienic and nuisance pest (Rust et al., 1995), whereas *B. nipponica* mainly lives in litter on the forest floor and neither enters nor reproduces in human residences, seldom being regarded as a pest species (Asahina, 1991; Harunari et al., 2007).

We collected *B. nipponica* from litter on the forest floor in the AIST campus, which mainly consisted of 4th, 5th and 6th instar nymphs and also contained some 2nd and 3rd instar nymphs and adults, and attempted to maintain them in the laboratory. In the first trial, we kept the insects using the same rearing system as used for *B. germanica* (see Materials and Methods), and the insects declined and died within a month. In the second trial, we kept the insects in the rearing container with forest litter, and the insects survived for up to two months. In the third trial, the insects were kept in the rearing container with forest litter and fed with fresh vegetables or fruits (lettuce, banana, apple, etc.) in addition to the standard insect feed, and some insects survived over three months. During the rearing experiments, only one adult female produced an ootheca, from which we obtained 10 first instar nymphs. These results indicate that there is still much room for improvement in stable laboratory rearing of *B. nipponica*.

Comparison of bacteriocytes and *Blattabacterium* between *B. germanica* and *B. nipponica*

In this way, we managed to obtain some developmentally staged insects of *B. nipponica*, which were investigated in comparison with *B. germanica*. The fat bodies in the body cavity of *B. nipponica* were less developed in comparison with those of *B. germanica* (Fig. 1J, K). The different levels of the fat body development may be attributable to their different nutritional conditions (field-collected *B. nipponica* vs. fully fed *B. germanica*), inherent physiological/anatomical differences between the species, or both. Fluorescence microscopy, FISH and TEM visualized rod-shaped symbiont cells and symbiont-harboring bacteriocytes within the visceral fat bodies of *B. nipponica*, which were similar to those in *B. germanica* (Fig. 1L–N). Molecular phylogenetic analysis showed that the *Blattabacterium* symbiont of *B. nipponica* is the closest to the *Blattabacterium* symbiont of *B. germanica* (Fig. 2), confirming the morphological and taxonomic affinity of the wild and pest *Blattella* species. FISH of whole body sections throughout the postembryonic developmental course of *B. nipponica* revealed that the distribution patterns of the bacteriocytes are quite similar to those in *B. germanica*: single bacteriocytes are scattered among adipocytes within the visceral fat bodies in the abdomen (Fig. 6A–F). Whole-mount FISH of first instar nymphs clearly visualized the three-dimensional distribution patterns of the bacteriocytes within the abdomen, which looked substantially similar between *B. germanica* and *B. nipponica* (Fig. 7).

Bacteriocytes and *Blattabacterium* in other cockroach species, *Periplaneta japonica*, *Periplaneta fuliginosa*, *Blatta lateralis* and *Blaptica dubia*

In addition to *B. germanica* and *B. nipponica*, we inspected the localization patterns of *Blattabacterium*-harboring bacteriocytes in diverse cockroaches, namely *Periplaneta japonica*, *Periplaneta fuliginosa*, *Blatta lateralis* and *Blaptica dubia*. *Blattabacterium* symbionts were phylogenetically identified from all the cockroach species (Fig. 2). FISH of whole body sections and whole-mount FISH of dissected fat bodies revealed that, despite their different body sizes and distinct morphological features, the distribution

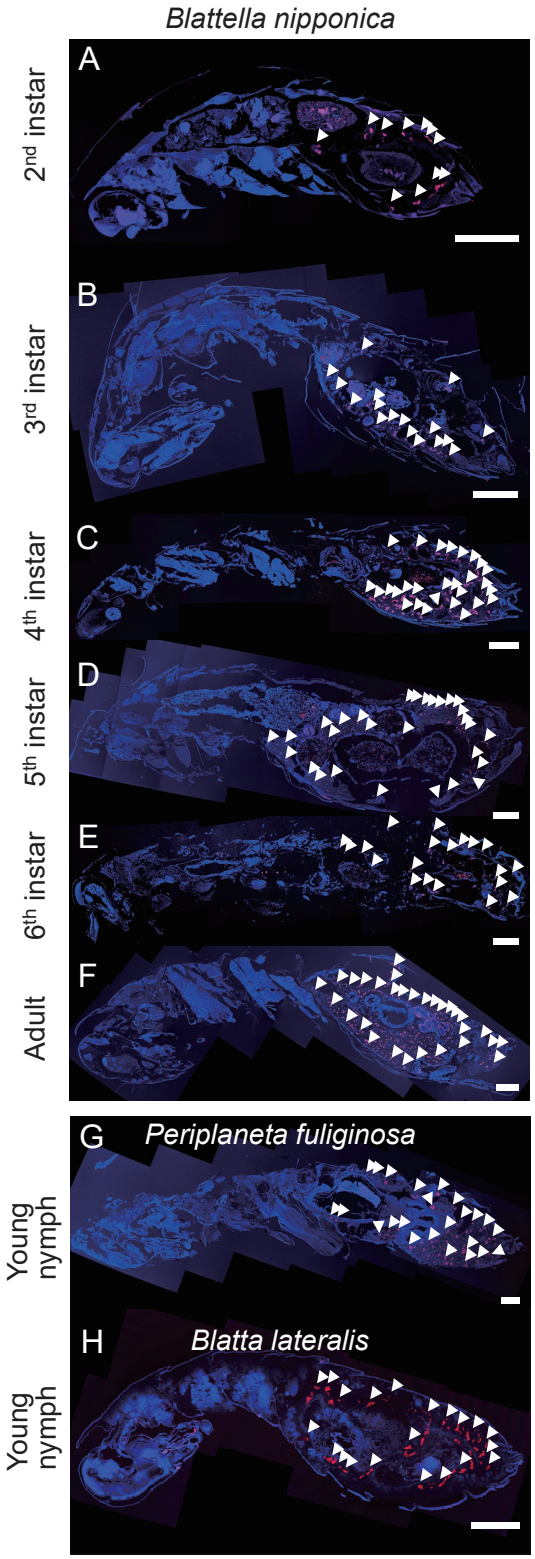

Fig. 6. Distribution of bacteriocytes in *B. nipponica* and other cockroach species. **(A)** A second instar nymph, **(B)** a third instar nymph, **(C)** a fourth instar nymph, **(D)** a fifth instar nymph, **(E)** a sixth instar nymph, and **(F)** an adult of *B. nipponica*. **(G)** A young nymph of *Periplaneta fuliginosa*. **(H)** A young nymph of *Blatta lateralis*. FISH images of sagittal whole body sections are displayed (left, head; right, abdomen; top, dorsal; bottom, ventral), in which bacteriocytes are visualized in red (arrowheads) and host nuclear DNA is counterstained in blue. Bars show 500 μm.

patterns of the bacteriocytes are strikingly similar across the diverse cockroach species: single bacteriocytes are scattered among adipocytes within the visceral fat bodies in the abdomen (Figs. 6G, H, 8). These results strongly favor the idea that the cockroach-*Blattabacterium* symbiotic association has been stably established and highly conserved over evolutionary time, which must date back to the common ancestor of the Blattodea some 300 million years ago in the Carboniferous (Grimaldi and Engel, 2005; Bell et al., 2007) and reflect the structurally intimate (Gier, 1936; Koch, 1949; Buchner, 1965), physiologically relevant (Cochran, 1985; Mullins, 2015), and evolutionarily co-speciating (Bandi et al., 1995; Lo et al., 2003) host-symbiont relationship.

Conclusion and perspective

In this study, using modern histological techniques including FISH, light microscopy and transmission electron microscopy, we present unprecedentedly detailed descriptions of the localization patterns of the *Blattabacterium*-harboring bacteriocytes during the postembryonic development of *B. germanica*. In classic microscopic studies such as Gier (1936) and Koch (1949), the infection processes of the sym-

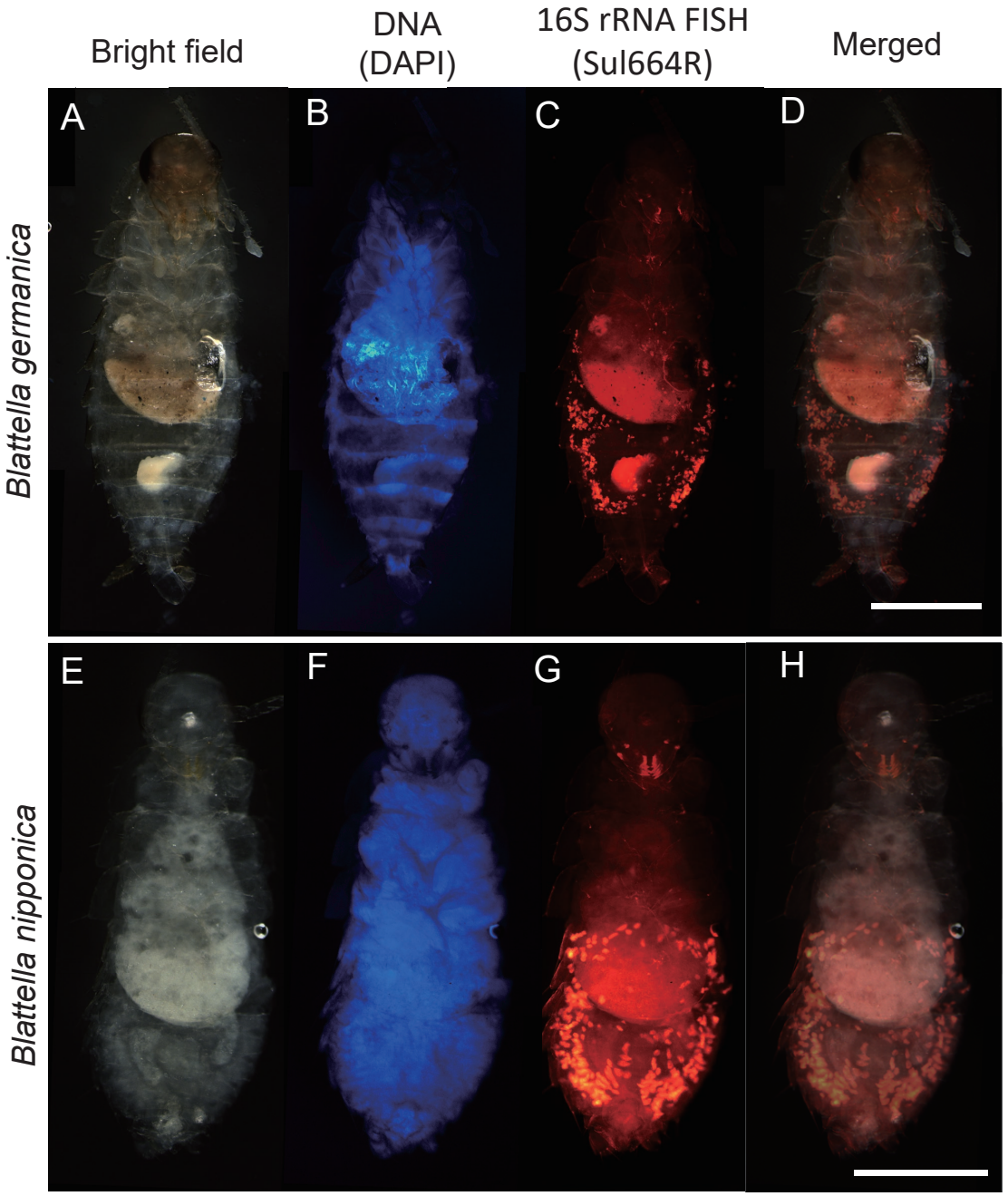

Fig. 7. Distribution of bacteriocytes in whole bodies of first instar nymphs of *B. germanica* **(A–D)** and *B. nipponica* **(E–H)** visualized by whole-mount FISH. From left to right are displayed bright field images, DNA staining fluorescence images, symbiont FISH fluorescence images, and merged fluorescence images. Bars show 1 mm.

biotic bacteria and the localization patterns of the bacteriocytes were described in detail during the embryogenesis of cockroaches, whereas equivalent comprehensive descriptions during the postembryonic development have been scarce. Our study fills the knowledge gap, and is therefore expected to be long referred to by future studies on the cockroach-*Blattabacterium* endosymbiosis.

We focused on *B. nipponica*, a wild cockroach species closely related to the cosmopolitan pest species *B. germanica*, which would potentially contribute to better understanding of the origin and the processes of urban pest evolution. However, our preliminary trials revealed that the laboratory rearing procedure for *B. nipponica* is still premature, with much room for improvement. Establishment of a stable laboratory rearing system for *B. nipponica* is anticipated, and we are currently working toward it. In Figure 7, whole body FISH of first instar nymphs of *B. germanica* and *B. nipponica* revealed substantially similar three-dimensional distribution patterns of the bacteriocytes. In these images, the bacteriocytes in the abdominal fat bodies may look somewhat larger and denser in *B. nipponica* than in *B. germanica* (cf. Fig. 7C, G), but, since we were able to histologically inspect only a few first instar nymphs of *B. nipponica* derived from a single ootheca, we need to examine more samples of *B. nipponica* reared under different nutritional conditions.

What biological aspects of *B. germanica* and *B. nipponica* are relevant to their pest and non-pest lifestyles despite their close phylogenetic relatedness is of both basic and applied interest. Generally speaking, urban pest insects like cockroaches may have evolved such traits as resistance to desiccation, loss of cold tolerance, altered feeding habit, loss of hibernation, and others (Schal and Hamilton, 1990; Bell et al., 2007; Mullins, 2015). It is expected, although

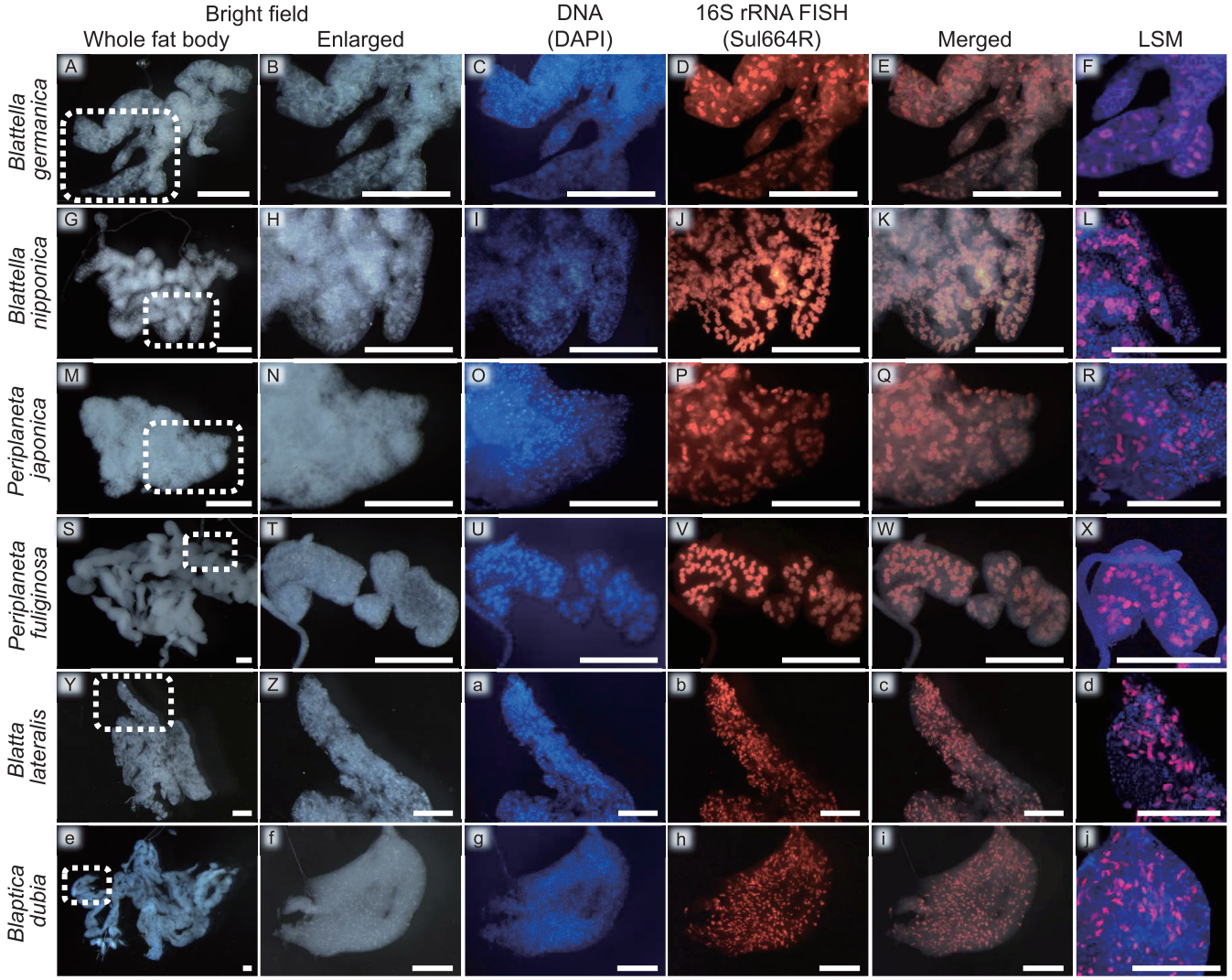

Fig. 8. Distribution of bacteriocytes in visceral fat bodies dissected from adult females of *B. germanica* **(A–F)**, *B. nipponica* **(G–L)**, *Periplaneta japonica* **(M–R)**, *Periplaneta fuliginosa* **(S–X)**, *Blatta lateralis* **(Y–d)**, and *Blaptica dubia* **(e–j)** visualized by whole-mount FISH targeting symbiont's 16S rRNA. From left to right are displayed bright field images of whole fat bodies, magnified bright field images of fat bodies (areas of dotted rectangles), DNA-staining fluorescence images, symbiont FISH fluorescence images, merged fluorescence images, and laser scanning microscopic images. Bars show 500 μm.

speculative, that non-pest cockroaches living in natural environments tend to be under nutrition-limited and nitrogen-deficient conditions, whereas pest cockroaches living in anthropic environments tend to be under nutrition- and nitrogen-rich conditions. If so, the *Blattabacterium* endosymbiont may be involved in the host's nitrogen metabolism differently between *B. germanica* and *B. nipponica*, to which our future studies will be directed. In this study, we adopted uric acid-removing histological procedures (see Materials and Methods) to obtain clear histological data on the distribution patterns of the bacteriocytes. Next, we will also focus on the urocytes accumulating uric acid crystals, which are structurally associated with the bacteriocytes and must play a pivotal role in cockroach's nitrogen metabolism and recycling (Cochran, 1985; Bell et al., 2007; Mullins, 2015).

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

TN and TF designed the study. TN performed most of the experiments. GO and MM assisted with DNA sequencing and molecular phylogenetic work. MM and RK assisted with the histological work. XYM performed transmission electron microscopy. TN and TF wrote the paper.

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