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Huge Symbiotic Organs in Giant Scale Insects of the Genus *Drosicha* (Coccoidea: Monophlebidae) Harbor Flavobacterial and Enterobacterial Endosymbionts

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Giant scale insects (*Drosicha*: Coccoidea: Monophlebidae) were investigated for their symbiotic organs and bacterial endosymbionts. Two types of bacterial 16S rRNA gene sequences, flavobacterial and enterobacterial, were consistently detected in *D. corpulenta* and *D. pinicola*. The former sequences formed a compact clade in the *Bacteroidetes*, allied to the symbionts of cushion and armored scales. The latter sequences formed a robust clade in the γ-*Proteobacteria*, allied to enteric bacteria like *Enterobacter aerogenes* and *Escherichia coli*. Another type of 16S sequence derived from *Wolbachia* was also detected in *D. pinicola*. In-situ hybridization demonstrated that the flavobacterial and enterobacterial symbionts were localized in a pair of huge bacteriomes in the abdomen, the former in uninucleated peripheral bacteriocytes and the latter in syncytial central bacteriocytes. Electron microscopy confirmed the endocellular locations of the pleomorphic flavobacterial symbiont and the rod-shaped enterobacterial symbiont, and also revealed the location and fine structure of the *Wolbachia* symbiont in *D. pinicola*. Infection frequencies of the flavobacterial and enterobacterial symbionts were consistently 100% in populations of *D. corpulenta* and *D. pinicola*, while the *Wolbachia* symbiont exhibited 0% and 100% infection frequencies in *D. corpulenta* and *D. pinicola*, respectively. Neither the flavobacterial symbiont nor the enterobacterial symbiont exhibited AT-biased nucleotide composition or accelerated molecular evolution. The huge bacteriomes of *Drosicha* giant scales would provide a useful system for investigating biochemical, physiological, and genomic aspects of the host-symbiont and symbiont-symbiont interactions.

Key words: *Drosicha*, giant scale, endosymbiont, bacteriome, bacteriocyte, symbiotic system, evolution

INTRODUCTION

Homopteran insects, including aphids, scale insects, whiteflies, psyllids, planthoppers, cicadas, and others, have needle-like mouthparts and feed exclusively on plant sap throughout their life. Plant sap is nutritionally unbalanced and difficult to utilize for most animals. Although rich in carbohydrates, mainly in the form of sucrose, plant sap contains very tiny amounts of lipids and proteins. While most lipids can be synthesized from carbohydrates, proteins cannot in the absence of nitrogenous precursors such as essential amino acids. Although some amino acids are present in plant sap, they are mostly nonessential. Homopteran insects generally rely on endosymbiotic microorganisms to supply essential amino acids and other nutrients, and can thereby subsist solely on specialized food sources, and some of them are among the most serious agricultural pests (Douglas, 2003; Baumann, 2005).

Scale insects (Coccoidea) are related to aphids (Aphidoidea), whiteflies (Aleyrodoidea), and psyllids (Psylloidea), constituting the suborder Sternorrhyncha in the order Hemiptera. While the primary symbionts are uniform and evolutionarily stable in aphids (with *Buchnera*), whiteflies (with *Portiera*), and psyllids (with *Carsonella*) (Baumann, 2005), the endosymbionts of scale insects are quite diverse: some harbor bacterial endosymbionts, whereas others are associated with yeast-like fungal endosymbionts, and their symbiotic cells, tissues, and organs often vary within and between lineages (Buchner, 1965). Thus far, microbiological characterization of the endosymbionts has been restricted to a limited number of scale insect groups: the β-proteobacterial (=*Tremblaya princeps*) and γ-proteobacterial endosym-

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bionts of mealybugs in Family Pseudococcidae (Munson et al., 1992; Kantheti et al., 1996; Fukatsu and Nikoh, 2000; von Dohlen et al., 2001; Thao et al., 2002; Baumann and Baumann, 2005; Downie and Gullan, 2005; Kono et al., 2008), and the flavobacterial endosymbionts of armored scales in Family Diaspididae, the cushion scales (*Icerya* spp.) in Family Monophlebidae, and a felt scale in Family Eriococcidae (Gruwell et al., 2005, 2007; Zchori-Fein et al., 2005).

The giant scales of the genus *Drosicha* belong to Family Monophlebidae, which constitutes a group distinct from but allied to the cushion scales (*Icerya* spp.). Two species of giant scales are commonly found in Japan. The large giant scale, *D. corpulenta*, lives on oaks and other broad-leaved trees and is among the world largest scale insects; mature unwinged adult females are up to 2 cm in body length, while adult males are winged and smaller in size (Fig. 1). The pine giant scale, *D. pinicola*, lives on pine trees and is much smaller; mature adult females are about 8 mm in body length. There were several early histological works on the endosymbiotic system of giant scales. Kitao (1928) described that *D. corpulenta* harbors yeast-like symbionts endocellularly in bacteriocytes, while the adjacent large syncytium is filled with pigment granules, although this interpretation soon turned out to be erroneous. Walczuch (1932) observed a pair of huge bacteriomes in the abdomen of *Monophlebus* spp., giant scales allied to *Drosicha* spp., wherein two types of bacteria are harbored in the bacteriocytes and syncytium, respectively. Buchner (1969) examined the bacteriomes of several *Drosicha* and *Monophlebus* species, and confirmed the presence of different symbiotic bacteria in the bacteriocytes and syncytium. The microbial nature of these endosymbionts, however, was unknown. In this study, we characterized the endosymbiotic bacteria of *Drosicha* giant scales microbiologically using molecular, phylogenetic, and histological techniques.

Fig. 1. A mating pair of the giant scale *Drosicha corpulenta*. The male is slender and winged, whereas the female is large and unwinged. Scale bar: about 1 cm. (Photo courtesy of N. Yamashiro).

MATERIALS AND METHODS

Materials

Insect samples used in this study are listed in Table 1. Upon collection, some insects were dissected with fine forceps under a binocular microscope to isolate bacteriomes. Other insects were immediately placed in acetone-filled glass vials and preserved at room temperature until molecular analyses (Fukatsu, 1999).

Molecular biological procedures

Each sample was crushed and digested in a 1.5-ml plastic tube with a lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.1 M NaCl, 0.5% sodium dodecyl sulfate, 0.2 mg/ml proteinase K) at 56°C overnight. DNA was extracted with phenol-chloroform, precipitated with ethanol, dried, and dissolved in TE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). Bacterial 16S rRNA gene fragments were amplified from the DNA samples by PCR using the following primer sets and conditions. A 1.5-kb fragment was amplified with primers 16SA1 (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16SB1 (5'-TAC GGY TAC CTT GTT ACG ACT T-3') under conditions of 95°C for 10 min followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 2 min (Fukatsu and Nikoh, 1998). A 1.1-kb fragment was amplified with primers EUB338F (5'-ACT CCT ACG GGA GGC AGC-3'), a sequence complementary to oligonucleotide probe EUB338 used to detect eubacterial 16S rRNA (Amann et al., 1990), and 16SB1 under conditions of 95°C for 10 min followed by 35 cycles of 95°C for 20 sec, 61°C for 20 sec, and 72°C for 2 min. A 1.5-kb fragment was amplified with primers 16SA1 and gma16S-R1 (5'-GTG ATT CAT GAC TGG GGT G-3') under conditions of 95°C for 10 min followed by 35 cycles of 95°C for 10 sec, 58°C for 30 sec, and 72°C for 2 min. A 0.6-kb fragment of the *wsp* gene from *Wolbachia* was amplified with primers wspF (5'-GGG TCC AAT AAG TGA TGA AGA AAC-3') and wspR (5'-TTA AAA CGC TAC TCC AGC TTC TGC-3') under conditions of 95°C for 10 min followed by 35 cycles of 95°C for 20 sec, 55°C for 20 sec, and 72°C for 1 min (Kondo et al., 2002). PCR products were cloned, genotyped by means of restriction fragment length polymorphisms, and sequenced essentially as previously described (Fukatsu and Nikoh, 1998).

Table 1. Insect samples used in this study, and their infection with flavobacterial, enterobacterial, and *Wolbachia* symbionts.

Species	Collection locality[1]	Host plant	Collection date	Collector	Infection rate			
					Stage	F-symbiont[2]	E-symbiont[3]	*Wolbachia* sp.
Drosicha corpulenta	Tsukuba, Ibaraki	*Quercus serrata*	May 8th, 2008	Y. Matsuura	Adult female	100% (27/27)	100% (27/27)	0% (0/27)
					Egg	100% (72/72)	100% (72/72)	0% (0/72)
	Ueno, Tokyo	*Lithocarpus edulis*	June 9th, 1996	T. Fukatsu	Adult female	100% (3/3)	100% (3/3)	0% (0/3)
	Uozu, Toyama	*Liquidambar styraciflua*	May 10th, 1998	T. Fukatsu	Adult female	100% (8/8)	100% (8/8)	0% (0/8)
Drosicha pinicola	Tsukuba, Ibaraki	*Pinus densiflora*	May 8th, 2008	Y. Matsuura	Adult female	100% (18/18)	100% (18/18)	100% (18/18)
					Adult male	100% (4/4)	100% (4/4)	100% (4/4)

[1] All the localities are in Japan.
[2] Flavobacterial symbiont.
[3] Enterobacterial symbiont.

Molecular phylogenetic analysis

Multiple alignments of nucleotide sequences were generated with the Vector NTI Advance 10.3.1 (Invitrogen). The alignments were then inspected and

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corrected manually with the GeneDoc 2.6.002 (Nicholas et al., 1997) to remove ambiguously aligned nucleotide sites. Phylogenetic analyses were conducted by three methods: maximum parsimony (MP), maximum likelihood (ML), and Bayesian analysis (BA). MP trees were constructed with the MEGA 4.0 (Tamura et al., 2007). In the analysis, all sites and character changes were weighed equally. ML and BA trees were created with PhyML 3.0 (Guindon and Gascuel, 2003) and MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003), respectively. We selected the GTR+I+G model for phylogenetic analyses with 16S, and the HKY+I model for analysis with *wsp*, on the basis of the Akaike information criterion estimated with Modeltest 3.7 (Posada and Crandall, 1998). Bootstrap values were determined from 100 replicates in the MP and ML analyses. In the BA analysis, 3750 trees were obtained for each analysis (ngen=500,000, samplefreq=100, burn in=1250) and used to construct a 50% majority-rule consensus tree.

Relative rate test

A relative rate test was performed with RRTree (Robinson-Rechavi and Huchon, 2000) on the basis of genetic distances estimated under Kimura's two-parameter model (Kimura, 1980). For the flavobacterial and enterobacterial 16S sequences, 1147 and 1349 unambiguously aligned nucleotide sites were subjected to the analysis, respectively.

Diagnostic PCR

A 0.2-kb fragment of the 16S rRNA gene from the flavobacterial symbiont was amplified with primers DcFlv1079F (5'-AGG TGT TGG GTT AAG TCC TGA-3') and DcFlv1248R (5'-CCA GTG GCT TCT CTC TGT A-3'). A 0.4-kb fragment of 16S from the enterobacterial symbiont was amplified with primers DcEnt628F (5'-AAC TGC ATT CGA GAC TGG T-3') and DcEnt1017R (5'-CCC GAA GGC ACC AAA GG-3'). A 0.6-kb fragment of *wsp* from the *Wolbachia* symbiont was amplified with the primers wspF and wspR. These PCR reactions were performed under conditions of 95°C for 10 min followed by 35 cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 1 min, with negative and positive control samples. To confirm the quality of template DNA in the samples, a 0.65-kb fragment of the insect 18S rRNA gene was amplified with primers 2880 (5'-CTG GTT GAT CCT GCC AGT AG-3') (Tautz et al., 1988) and B (5'-CCG CGG CTG CTG GCA CCA GA-3') (von Dohlen and Moran, 1995) under conditions of 95°C for 10 min followed by 30 cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 1 min.

Fluorescence in-situ hybridization

Fluorescence in-situ hybridization was performed essentially as previously described (Koga et al., 2009) with oligonucleotide probe Cy5-DcFlv1410 (5'-Cy5-ATA CCT CCG ACT TCC AGG A-3'), which targeted 16S rRNA of the flavobacterial symbiont, and Cy3-DcEnt1248 (5'-Cy3-GAG GTC GCT TCT CTT TG-3'), which targeted 16S rRNA of the enterobacterial symbiont.

For whole-mount in-situ hybridization, female adult insects were dissected and thoroughly washed in phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4 [pH 7.4]), and preserved in acetone until use. Male adult insects were decapitated to facilitate the infiltration of reagents, and preserved in acetone. The preserved insect samples were dissected and thoroughly washed in 80% ethanol, and fixed in Carnoy's solution (6:3:1 ethanol:chloroform:acetic acid). After an overnight fixation, the tissues were treated with 6% hydrogen peroxide in 80% ethanol for several weeks to quench autofluorescence from the tissues (Koga et al., 2009). After thorough washing with absolute ethanol and PBST buffer (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4 [pH 7.4], 0.2% Tween20), the samples were incubated with hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 30% formamide) three times for 5 min each. The prehybrized samples were hybridized in 100 nM solutions of each of the probes Cy5-DcFlv1410 and Cy3-DcEnt1248 in hybridization buffer overnight. Nuclei of the host cells were counterstained with 4 μM 4', 6-diamidino-2-phynylindole (DAPI). After thorough washing with PBST, the samples were mounted in Slowfade antifade solution (Invitrogen) and observed under an Axiophot epifluorescence microscope (Carl Zeiss) or a Pascal 5 laser confocal microscope (Carl Zeiss). The specificity of the hybridization signals was confirmed by the following control experiments: no-probe control, RNase digestion control, and competitive suppression control with excess unlabelled probes.

For in-situ hybridization of sectioned tissues, the preserved samples were washed with 80% ethanol and treated with 6% hydrogen peroxide in 80% ethanol to quench autofluorescence. The samples were thoroughly washed with absolute ethanol, dehydrated and cleared through an ethanol-xylene series, embedded in paraffin, and processed into 4-μm serial tissue sections on a rotary microtome. The tissue sections were mounted on silane-coated glass slides, dewaxed through a xylene-ethanol series, and dried. The tissue preparations were incubated in about 150 μl of hybridization buffer containing 100 nM each probe, covered with a coverslip, and incubated in a humidified chamber at room temperature overnight. After thorough washing with Tris-buffered saline (20 mM Tris-HCl [pH 7.4], 0.15 M NaCl), the samples were mounted in Slowfade antifade solution (Invitrogen) supplemented with either 4 μM DAPI or 0.25 μM SYTOX Green (Invitrogen). The rest of the procedure was performed in the same manner as the whole-mount in-situ hybridization.

Electron microscopy

Fresh insects were dissected with fine forceps in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde, and isolated bacteriomes were prefixed in the fixative at 4°C overnight and postfixed in 2% osmium tetroxide at 4°C for 90 min. After dehydration through an ethanol series, the materials were embedded in Spurr resin (Nisshin-EM). Ultrathin sections were made on an Ultracat-N ultramicrotome (Leichert-Nissei), mounted on collodion-coated copper meshes, stained with uranyl acetate and lead citrate, and observed under a Model H-7000 transmission electron microscope (Hitachi).

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB491196–AB491203 and AB492154.

RESULTS

General observation of the symbiotic organ

Fig. 2A shows the internal organs of a dissected adult female *D. corpulenta*. In the central abdomen, there were large white ovaries and red Malphigian tubules. On both sides of the abdominal body cavity, there were symbiotic organs consisting of around six large lobes, the bacteriomes. The bacteriome lobes were connected anteroposteriorly to each other and often reached 5 mm in length. Light microscopy of tissue sections revealed that the bacteriomes consisted of three cellular components: large, syncytial central bacteriocytes located at the center of each lobe; smaller, uninucleate peripheral bacteriocytes surrounding the central bacteriocytes; and thin sheath cells located on the outer surface of the bacteriomes as well as intercalating the bacteriocytes (data not shown). Symbiont-like particles were observed in the cytoplasm of the central and peripheral bacteriocytes (data not shown). The same configuration of the symbiotic organs was observed in *D.*

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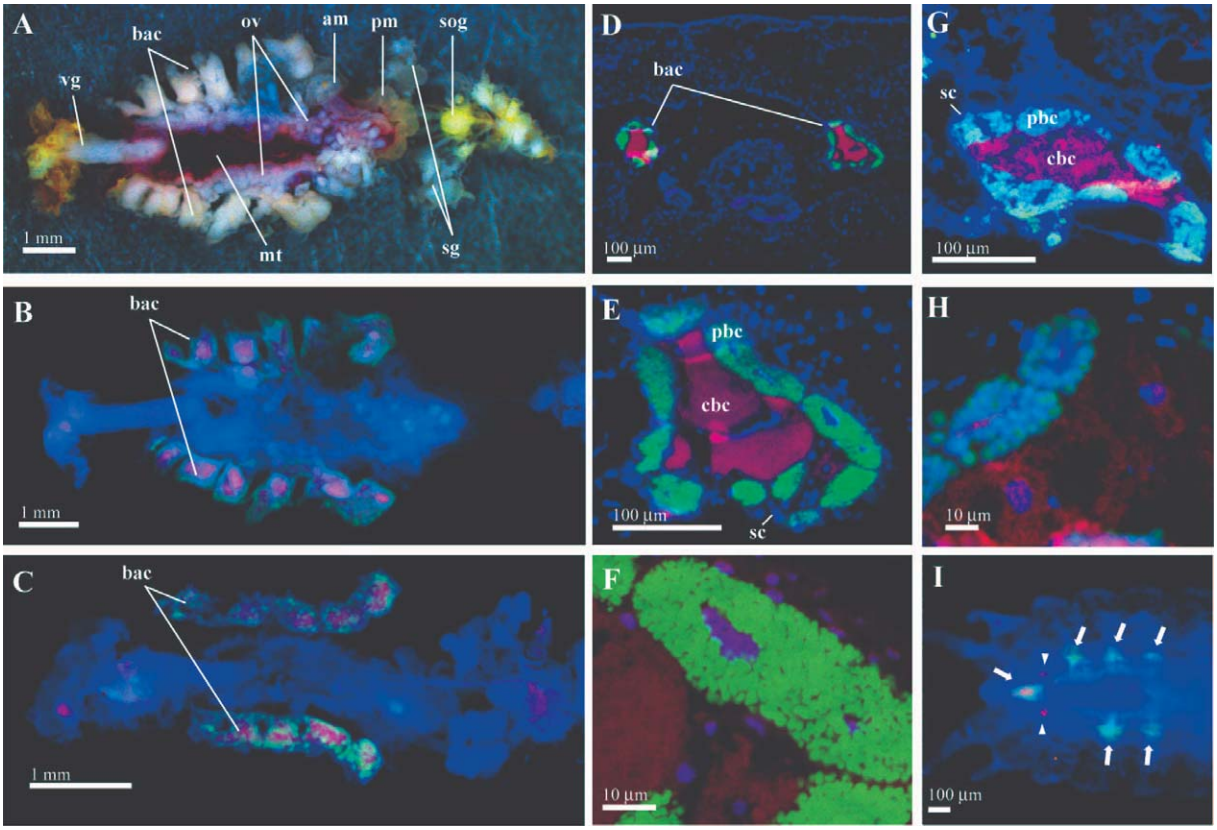

Fig. 2. Internal tissues and organs of *Drosicha* giant scales, and localization of the flavobacterial (green) and enterobacterial (red) symbionts visualized by fluorescence in-situ hybridization. **(A)** Dorsal view of the dissected whole body of an adult female *D. corpulenta*. A pair of large lobed bacteriomes (3–5 mm in length) is located on both sides of the abdomen. **(B)** Localization of the flavobacterial symbiont and the enterobacterial symbiont in the bacteriome lobes of *D. corpulenta*. **(C)** Co-localization of the flavobacterial and enterobacterial symbionts in the bacteriome lobes of *D. pinicola*. **(D)** Localization of the flavobacterial and enterobacterial symbionts in the bacteriome lobes of *D. corpulenta*. **(E)** Enlarged image of a bacteriome lobe of *D. corpulenta* harboring the flavobacterial and enterobacterial symbionts in the peripheral and central bacteriocytes, respectively. **(F)** Enlarged image of bacteriocytes of *D. corpulenta*, wherein symbiont cells are evident in the cytoplasm. **(G)** Enlarged image of a bacteriome lobe of *D. pinicola*. **(H)** Enlarged image of bacteriocytes of *D. pinicola*. **(I)** Localization of the flavobacterial and enterobacterial symbionts in an adult male *D. pinicola*. Arrows and arrowheads indicate signals from the flavobacterial and enterobacterial symbionts, respectively. (A–C) and (I) are images of whole mounts; (D–H) are images of sectioned tissue. Abbreviations: am, anterior midgut; bac, bacteriome; cbc, central bacteriocyte; mt, Malpighian tubule; ov, ovary; pbc, peripheral bacteriocyte; pm, posterior midgut; sc, sheath cell; sg, salivary gland; sog, suboesophageal ganglion; vg, vagina.

pinicola, with the bacteriomes consisting of around five lobes and reaching 2 mm in length (data not shown).

Bacterial 16S rRNA gene sequences from *D. corpulenta*

From an adult female of *D. corpulenta* from Tsukuba, 24 clones of the 16S rRNA gene fragment amplified with primers 16SA1 and 16SB1 were sequenced and yielded 22 identical flavobacterial sequences and two identical γ-proteobacterial sequences. These sequences were consistently identified from five more insect individuals from Tsukuba, three from Ueno, and three from Uozu. BLASTN searches against the DNA databases identified the highest hits for the flavobacterial sequence as a bacterial symbiont from the cushion scale *Icerya brasiliensis* (96.3% sequence similarity [1239/1286], accession number DQ133551), and for the γ-proteobacterial sequence, as an uncultured bacterium from the antlion *Myrmeleon mobilis* (98.5% [1421/1443], DQ068866).

Bacterial 16S rRNA gene sequences from *D. pinicola*

From an adult female of *D. pinicola* from Tsukuba, 24 clones of the 16S fragment amplified with the primers 16SA1 and 16SB1 were sequenced and yielded 18 identical flavobacterial sequences and six identical α-proteobacterial sequences. These sequences were consistently detected from six more insect individuals from Tsukuba. BLASTN searches against the DNA databases retrieved the highest hits for the flavobacterial sequence as symbiont from the cushion scale *Icerya brasiliensis* (96.3% [1239/1286], DQ133551) and for the α-proteobacterial sequence as a *Wolbachia* symbiont from the chrysomelid beetle *Diabrotica lemniscata* (99.2% [1416/1427], AY007547). PCR with primers EUB338F and 16SB1 amplified a γ-proteobacterial sequence very similar to that identified from *D. corpulenta*. All eight clones sequenced represented the same γ-proteobacterial sequence, but no flavobacterial sequence was obtained, probably because EUB338F contained mismatches to the flavobacterial sequence. On the basis of

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the sequence determined, we designed a new, specific reverse primer, gma16S-R1, upstream of 16SB1. PCR with primers 16SA1 and gma16S-R1 consistently yielded the γ-proteobacterial sequence from 10 individuals of *D. pinicola*. A BLASTN search against the DNA databases retrieved the highest hit for the γ-proteobacterial sequence as *Pantoea agglomerans* (98.2% [1417/1443], AM184290).

Molecular phylogenetic analysis of flavobacterial sequences

Fig. 3 shows the phylogenetic relationships among the flavobacterial 16S rRNA gene sequences from *D. corpulenta* and *D. pinicola* and the representative flavobacterial sequences. The sequences from the giant scales formed a highly supported clade with the symbiont sequences from the cushion scales (*Icerya* spp.). Note that *Drosicha* spp. and *Icerya* spp. belong to the same family Monophlebidae. The symbiont sequence from *Cryptococcus ulmi* of Family Eriococcidae was placed just outside the clade. These sequences constituted a sister clade to that containing the symbiont sequences from armored scale insects of Family Diaspididae (*Uzinura diaspidicola*). The monophlebid-eriococcid-diaspidid symbiont sequences further formed a sister clade to the clades comprising the male-killing symbiont sequences from lady beetles, the symbiont sequences from cockroaches and termites (*Blattabacterium* spp.), and the symbiont sequences from cicadas and planthoppers (*Sulcia mulleri*). Sequences from free-living flavobacteria were placed outside the large clade of flavobacterial insect symbionts. We designate this type of *Drosicha* symbionts as "flavobacterial symbionts".

Molecular phylogenetic analysis of γ-proteobacterial sequences

Fig. 4 shows the phylogenetic relationships among the γ-proteobacterial 16S rRNA gene sequences from *D. corpulenta* and *D. pinicola* and representative γ-proteobacterial sequences. The symbiont sequences from the giant scales formed a robust clade, and were allied to free-living γ-proteobacteria such as *Enterobacter aerogenes* and *Escherichia coli*. No γ-proteobacterial insect symbiont sequences exhibited close phylogenetic affinity to the *Drosicha* symbiont sequences. We designate this type of *Drosicha* symbionts as "enterobacterial symbionts".

Molecular phylogenetic analysis of *Wolbachia* sequence

Fig. 5 shows the phylogenetic placement of the *wsp* sequence from *Wolbachia* from *D. pinicola*. This sequence was closely related to the *Wolbachia* sequence from the sand fly *Phlebotomus papatasi*, and was placed in *Wolbachia* supergroup A.

Prevalence of flavobacterial, enterobacterial, and *Wolbachia* symbionts in host populations

Diagnostic PCR surveys revealed 100% infection frequencies for the flavobacterial and enterobacterial symbionts in populations of *D. corpulenta* and *D. pinicola*. The *Wolbachia* symbiont exhibited 0% and 100% infection frequencies in populations of *D. corpulenta* and *D. pinicola*, respectively (Table 1).

Localization of flavobacterial and enterobacterial symbionts

Whole-mount in-situ hybridization of adult females of *D. corpulenta* revealed that the flavobacterial and enterobacterial symbionts are localized peripherally and centrally in the bacteriome

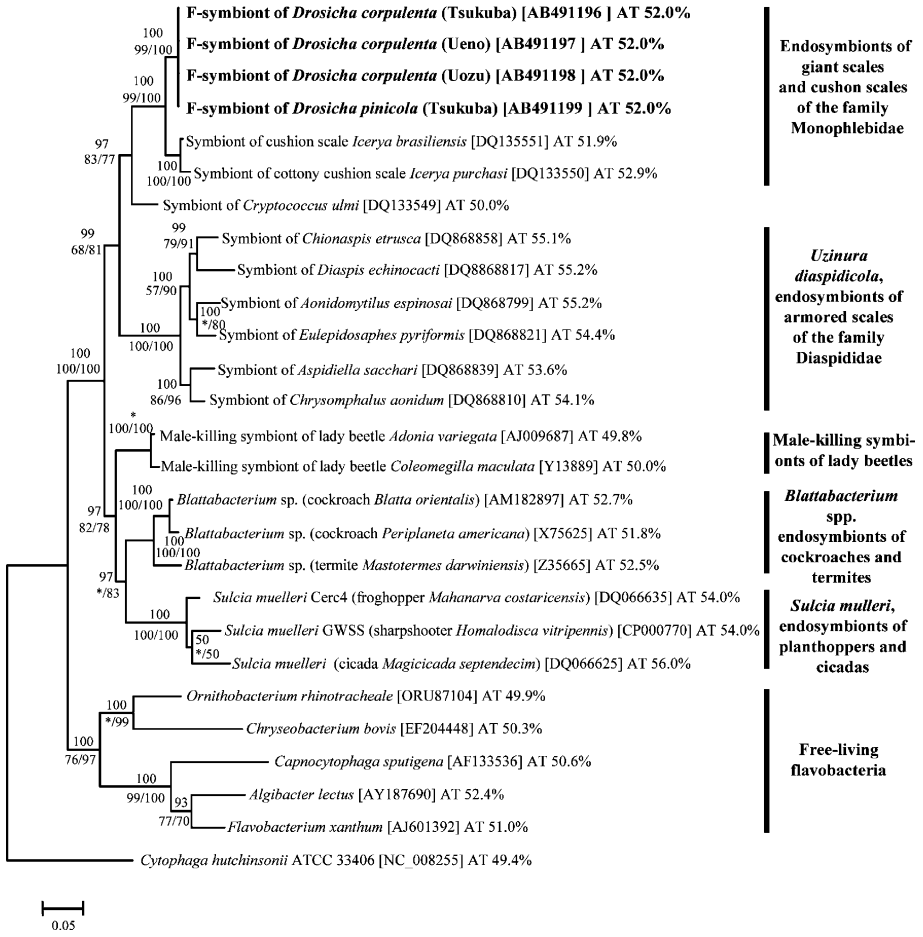

Fig. 3. Molecular phylogeny including the flavobacterial symbionts from *Drosicha* giant scales and allied symbiotic and free-living flavobacteria, inferred from 16S rRNA gene sequences. In all, 1149 aligned nucleotide sites were analyzed. The Bayesian (BA) tree is shown, but the MP and ML analyses gave substantially the same results (data not shown). Support values are indicated near nodes, with posterior probabilities from the BA shown above bootstrap values from the MP/ML analyses. Asterisks indicate support values lower than 50%; sequences from *Drosicha* giant scales are indicated by bold font; collection localities are in parentheses; sequence accession numbers are in brackets; and the AT content (%) of each sequence is given. F-symbiont, flavobacterial symbiont.

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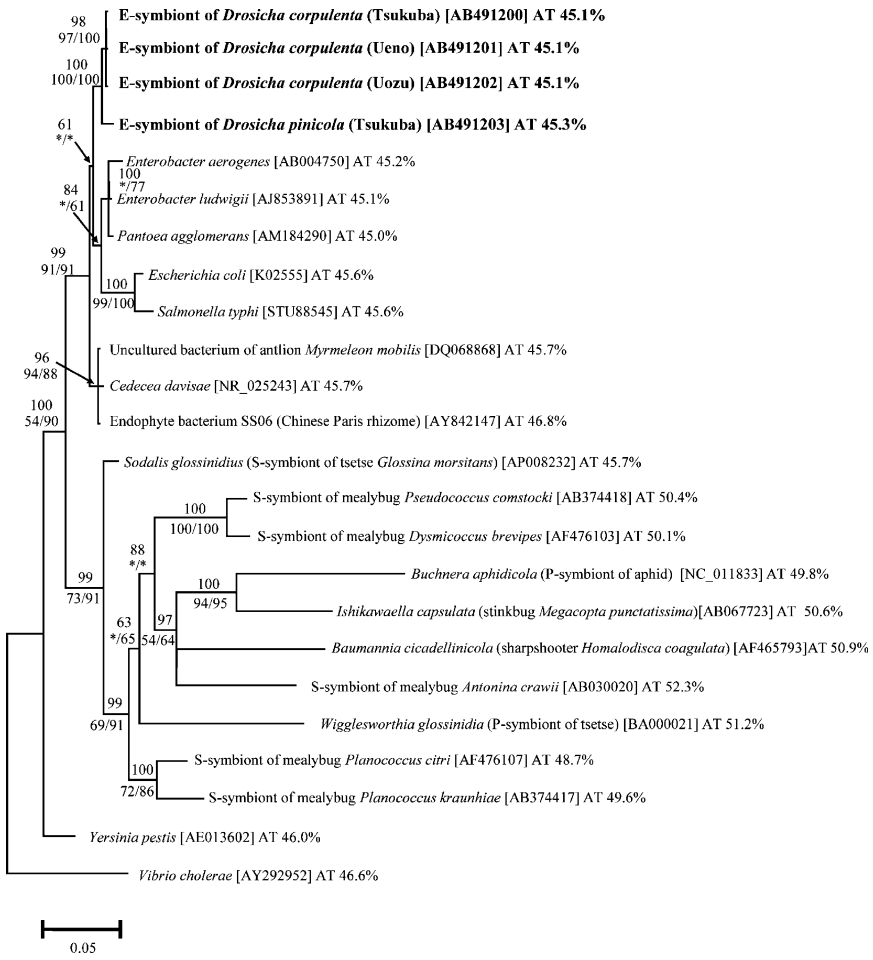

Fig. 4. Molecular phylogeny including the enterobacterial symbionts from *Drosicha* giant scales and allied symbiotic and free-living γ-proteobacteria, inferred from 16S rRNA gene sequences. In all, 1338 aligned nucleotide sites were analyzed. The BA tree is shown, but the MP and ML analyses gave substantially the same results (data not shown). Support values are indicated near nodes, with the posterior probability from the BA analysis shown above the bootstrap values from the MP/ML analyse. Asterisks indicate support values lower than 50%; sequences from *Drosicha* giant scales are indicated by bold font; collection localities are in parentheses; sequence accession numbers are in brackets; and the AT content (%) of each sequence is given. E-symbiont, enterobacterial symbiont.

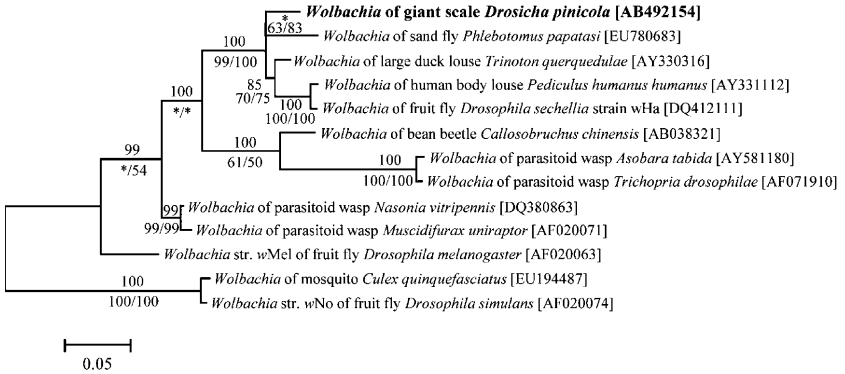

Fig. 5. Molecular phylogenetic placement of the *Wolbachia* symbiont from *Drosicha pinicola* in *Wolbachia* supergroup A, on the basis of *wsp* gene sequences. In all, 504 aligned nucleotide sites were analyzed. The BA tree is shown, but the MP and ML analyses gave substantially the same results (data not shown). Support values are indicated near nodes, with the posterior probability from the BA analysis shown above the bootstrap values from the MP/ML analyses. Asterisks indicate support values lower than 50%; the sequence from *D. pinicola* is indicated by bold font; sequence accession numbers are in brackets.

lobes, respectively (Fig. 2B). In-situ hybridization of tissue sections confirmed the peripheral and central locations of the flavobacterial and enterobacterial symbionts in the bacteriome lobes (Fig. 2D, E). The uninucleate peripheral bacteriocytes harbored the flavobacterial symbiont in their cytoplasm (Fig. 2E, F), whereas the syncytial central bacteriocytes housed the enterobacterial symbiont endocellularly (Fig. 2D, E). In adult females of *D. pinicola*, the same patterns of symbiont localization were observed (Fig. 2C, G, H). In adult males of *D. pinicola*, the flavobacterial and enterobacterial symbionts each exhibited a peculiar localization pattern in the abdomen (Fig. 2I).

Fine structure of the flavobacterial, enterobacterial, and *Wolbachia* symbionts

Fig. 6 shows transmission electron micrographs of the bacteriomes of *Drosicha* giant scales. In *D. corpulenta*, the flavobacterial symbiont was found in the peripheral bacteriocytes as large, pleomorphic bacterial cells with reduced cell wall, whereas the enterobacterial symbiont densely populated the central bacteriocytes as rod-shaped bacterial cells (Fig. 6A–C). In *D. pinicola*, the pleomorphic flavobacterial symbiont and the rod-shaped enterobacterial symbiont were similarly localized in the peripheral and central bacteriocytes, respectively (Fig. 6D–F). In *D. pinicola*, moreover, small rod-shaped bacterial cells of a different type densely populated the sheath cells (Fig. 6D, G) and were also observed in the bacteriocytes at lower densities (Fig. 6D, F). The small bacterial rods exhibited a well-developed cell wall (Fig. 6H). Judging from the molecular phylogenetic and diagnostic PCR data (Table 1; Fig. 5), the small bacteria were probably the *Wolbachia* symbiont.

Molecular evolution of the flavobacterial and enterobacterial symbionts

Relative rate tests detected no significant acceleration in molecular evolutionary rates in the 16S rRNA gene in the lineages of the flavobacterial and enterobacterial symbionts of *Drosicha* giant scales (Table 2). The 16S sequences from the symbionts exhibited no remarkable AT bias in nucleotide composition compared to the 16S sequences from their free-living relatives (Figs. 3, 4).

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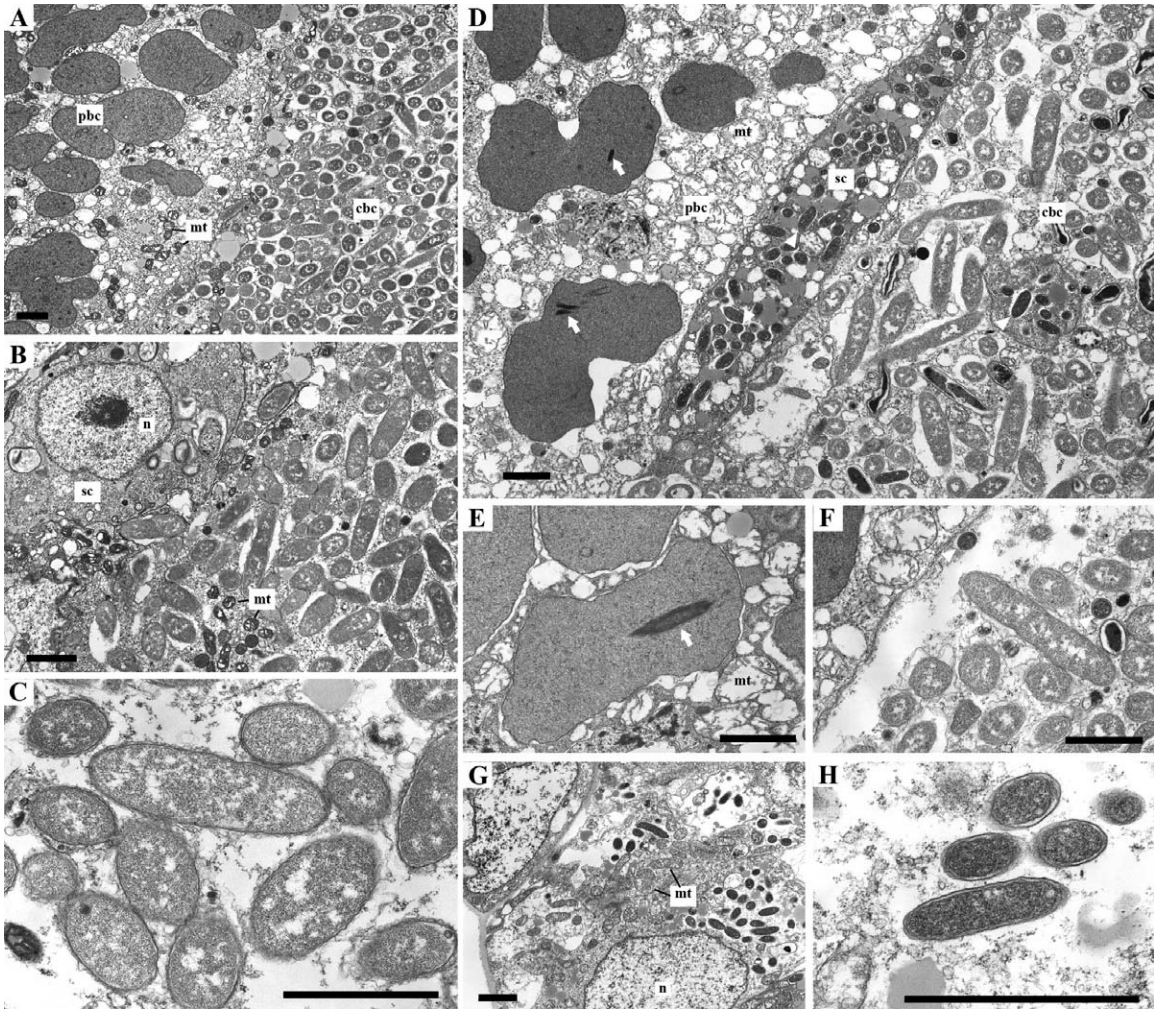

Fig. 6. Transmission electron micrographs of the bacterial symbionts of *Drosicha* giant scales. **(A)** Pleomorphic flavobacterial symbiont cells in a peripheral bacteriocyte (left) and rod-shaped enterobacterial symbiont cells in a central bacteriocyte (right) in *D. corpulenta*. **(B)** A central bacteriocyte in *D. corpulenta* filled with enterobacterial symbiont cells (right) and an adjacent sheath cell harboring few symbionts (left). **(C)** Enlarged image of enterobacterial symbiont cells in *D. corpulenta*. **(D)** Pleomorphic flavobacterial symbiont cells in a peripheral bacteriocyte (left), rod-shaped enterobacterial symbiont cells in a central bacteriocyte (right), and presumed *Wolbachia* symbiont cells in a sheath cell (center) of *D. pinicola*. Some *Wolbachia* cells are also seen in the central bacteriocyte. **(E)** Enlarged image of flavobacterial symbiont cells in *D. pinicola*. A crystalline body (arrow) is evident in a symbiont cell. **(F)** Enlarged image of enterobacterial symbiont cells in *D. pinicola*. **(G)** Presumed *Wolbachia* symbiont cells in sheath cells of *D. pinicola*. **(H)** Enlarged image of presumed *Wolbachia* cells. Abbreviations: cbc, central bacteriocyte; mt, mitochondrion; n, nucleus; pbc, peripheral bacteriocyte; sc, sheath cell. Arrows, crystalline bodies observed in flavobacterial symbiont cells. Bars, 2μm.

DISCUSSION

Here we identified two kinds of bacteria, the flavobacterial symbiont and the enterobacterial symbiont, from the giant scales *D. corpulenta* and *D. pinicola* (Figs. 3, 4). In-situ hybridization and electron microscopy demonstrated that these symbionts are specifically localized in different types of host cells in the bacteriomes: the pleomorphic flavobacterial symbiont in the uninucleated peripheral bacteriocytes and the rod-shaped enterobacterial symbiont in the syncytial central bacteriocytes (Figs. 2, 6). Diagnostic PCR surveys revealed 100% infection frequencies of the symbionts in natural host populations (Table 1). This study provides the first microbiological characterization of endosymbiotic bacteria from *Drosicha* giant scales.

Early histological works described two types of vertically transmitted symbiotic bacteria in the bacteriomes of several giant scales (Walczuch, 1932; Buchner, 1969). A recent electron microscopic study showed that numerous pleomorphic bacteria inhabit the bacteriocytes and oocytes of a giant scale together with another type of bacteria (Szklarzewicz et al., 2006). Recent molecular analyses indicated that *Icerya* spp., which are allied to *Drosicha* spp. in Family Monophlebidae, possess the flavobacterial symbiont (Gruwell et al., 2005; 2007). Our results presented here integrate previous data on the endosymbiosis of giant scales into a coherent picture.

Interestingly, endosymbiotic bacteria allied to the flavobacterial symbiont of *Drosicha* giant scales have been identified from cushion scales of Family Monophlebidae, a felt scale of Family Eriococcidae, and armored scales of Family Diaspididae (Fig. 3). It appears plausible that the flavobacterial symbionts represent the obligate primary symbionts of diverse

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Table 2. Relative rate tests comparing the molecular evolutionary rate of 16S rRNA gene sequences between the lineages of *Drosicha* symbionts and their free-living relatives.

Lineage 1	Lineage 2	Outgroup	K1[1]	K2[2]	Difference in distance[3]	Rate ratio[4]	*P* value[5]
F-symbionts of *Drosicha* scales[6]	Free-living flavobacteria[7]	*Cytophaga hutchinsonii*[8]	0.104	0.103	0.001	1.01	0.95
E-symbionts of *Drosicha* scales[9]	Free-living γ-proteobacteria[10]	*Yersinia pestis*[11]	0.015	0.023	–0.008	0.65	0.11

[1] Estimated mean distance between lineage 1 and the last common ancestor of lineages 1 and 2.
[2] Estimated mean distance between lineage 2 and the last common ancestor of lineages 1 and 2.
[3] K1-K2.
[4] K1/K2.
[5] *P*-values were generated with the program package RRTree (Robinson-Rechavi and Huchon, 2000). Kimura's two-parameter model (Kimura, 1980) was used to correct for multiple substitutions.
[6] Flavobacterial symbionts from *Drosicha corpulenta* (Tsukuba) [AB491196], *D. corpulenta* (Ueno) [AB491197], *D. corpulenta* (Uozu) [AB491198], and *D. pinicola* (Tsukuba) [AB491203].
[7] Free-living flavobacteria *Algibacter lectus* [AY187690], *Flavobacterium xanthum* [AJ601392], *Capnocytophaga sputigena* [AF133536], *Chryseobacterium bovis* [EF204448], and *Ornithobacterium rhinotracheale* [ORU87104].
[8] Genbank NC_008255.
[9] Enterobacterial symbionts from *Drosicha corpulenta* (Tsukuba) [AB491200], *D. corpulenta* (Ueno) [AB491201], *D. corpulenta* (Uozu) [AB491202], and *D. pinicola* (Tsukuba) [AB491203].
[10] *Escherichia coli* [K02555] and *Salmonella typhi* [STU88545].
[11] Genbank AE013602.

scale insects, and that the evolutionary origin of the endosymbiosis was quite ancient. It is also notable that allied flavobacterial symbionts have been reported from such phylogenetically distant insect groups as the male-killing symbionts of lady beetles, *Blattabacterium* spp. of cockroaches and termites, and *Sulcia mulleri* of planthoppers and cicadas (Fig. 3). These patterns suggest that a flavobacterial lineage evolved the ability to establish an endosymbiotic association with an insect a long time ago, and that this lineage has diversified into the flavobacterial insect endosymbionts that we currently observe. The pleomorphism and reduced cell wall of the flavobacterial symbionts (Fig. 6) might suggest an ancient origin of the endosymbiosis.

By contrast, no insect endosymbionts allied to the enterobacterial symbionts of *Drosicha* giant scales have been reported. The enterobacterial symbionts are related to free-living enteric bacteria (Fig. 4) and exhibit fine structures typical of common rod-shaped bacteria (Fig. 6). These results suggest that the enterobacterial symbionts are of relatively recent evolutionary origin, acquired later than the flavobacterial symbiont. Of course, the possibility cannot be excluded that the enterobacterial symbionts are actually present in allied scale insects but have simply not yet been identified.

Multiple endosymbionts coexist in a wide array of plant-sucking insects, including aphids (Fukatsu et al., 1998), mealybugs (Kono et al., 2008), whiteflies (Gottlieb et al., 2008), psyllids (Fukatsu and Nikoh, 1998), sharpshooters (Moran et al., 2003), and many others. The configuration of the endosymbiotic system of *Drosicha* giant scales is reminiscent of those of aphids and psyllids in that the primary symbiont is harbored in peripheral uninucleated bacteriocytes and the secondary symbiont is located in central syncytial bacteriocytes. The endosymbiotic bacteria of *Drosicha* giant scales look similar to those of sharpshooters in that the primary symbiont is a flavobacterium and the secondary symbiont is a γ-proteobacterium.

Genome analyses of the sharpshooter endosymbionts have suggested that the flavobacterial primary symbiont, *Sulcia mulleri*, provides the host with essential amino acids whereas the γ-proteobacterial secondary symbiont, *Baumannia cicadellinicola*, supplies vitamins and cofactors (Wu et al., 2006). Although the biological roles of the flavobacterial and enterobacterial symbionts in *Drosicha* giant scales are currently unknown, complementary metabolic and physiological processes like these may be operating in the insects that live exclusively on nutritionally unbalanced tree sap.

Obligate endosymbiotic γ-proteobacteria that have co-evolved with their host insects, such as *Buchnera* in aphids, *Wigglesworthia* in tsetse flies, *Carsonella* in psyllids and others, commonly exhibit peculiar genetic traits, including AT-biased nucleotide composition, an accelerated rate of molecular evolution, and significant genome reduction (Wernegreen, 2002). However, neither the flavobacterial nor the enterobacterial symbionts exhibited these evolutionary patterns in *Drosicha* giant scales (Figs. 3, 4; Table 2). As for the enterobacterial symbionts, the absence of the patterns might be attributable to a recent evolutionary origin. In addition to the flavobacterial symbionts of *Drosicha* giant scales, other ancient flavobacterial endosymbiont lineages such as *Blattabacterium* and *Sulcia* exhibit no AT-biased nucleotide composition (Fig. 3). Why the evolutionary patterns in the flavobacterial insect endosymbionts differ from those in the γ-proteobacterial insect endosymbionts is of interest and deserves future analyses.

In the pine giant scale *D. pinicola*, all individuals were infected with the *Wolbachia* symbiont, while no individuals of *D. corpulenta* were (Table 1). It appears plausible, although speculative, that the *Wolbachia* symbiont causes cytoplasmic incompatibility in *D. pinicola*, thereby attaining a prevalent infection in the host population. The concentrated *Wolbachia* infection in sheath cells (Fig. 6) is of interest, suggesting that the *Wolbachia* symbiont may somehow interact with the flavobacterial and enterobacterial symbionts in the same bacteriomes.

Two pieces of bacteriome as large as 5 mm in length can be prepared from a single adult female *D. corpulenta*. Even a single dissected bacteriome may be sufficient for the analysis of protein contents, measurement of enzymatic activities, tracer experiments, etc. Even from a single host insect, a sufficient quantity of endosymbiont DNA can be prepared for shotgun genome sequencing (Matsuura et al., unpublished data). We point out that the huge symbiotic organs of *Drosicha* giant scales would provide a useful model system for investigating biochemical, physiological and genomic aspects of the host-symbiont and symbiont-symbiont interactions.

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