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

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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 (Aleyrodoidae), 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 (=*Treblaya princeps*) and *γ*-proteobacterial endosym-
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., 2002). 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 anatomy of these endosymbiotic systems of giant scales. Kitao (1928) described that *D. corpulenta* harbors yeast-like symbionts enterobacterially 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.

**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 10 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 ACC-3'), a sequence complementary to oligonucleotide probe EUB338 used to detect eubacterial 16S rRNA (Amann et al., 1990), 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 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 GTC TGG CTG-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 TAC AAT AAG TGA TGG ACG AAC-3') and wspR (5'-TTA AAA CGG TAC TCC AGC TCC 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 (Kono 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).

**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
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, burnin=1250) and used to construct a 50% majority-rule consensus tree.

Relative rate test
A relative rate test was performed with RRTree (Robinson-Rechav and Buchon, 2000) on the basis of the 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 AGG A-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 presence of the symbiont, the samples were incubated with hybridization buffer (20 mM Tris-HCl [pH 7.4], 0.15 M NaCl), the samples were incubated in SlowFade anti-fluorescent solution (Invitrogen) supplemented with either 4 M DAPI or 0.25 M 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 (Nissin-EM). Ultrathin sections were made on an Ultratoc-N ultramicrotome (Leica-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).

RESULTS

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 TCT CCT TTG-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 Na2HPO4, 2.7 mM KH2PO4 [pH 7.4]), and preserved in acetone until use. Male adult insects were decapitated to facilitate the infiltration of reagents, and intercalating the bacteriocytes (data not shown). Symbiont-located on the outer surface of the bacteriomes as well as rounding the central bacteriocytes; and thin sheath cells posteriorly to each other and often reached 5 mm in length.

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 (Nissin-EM). Ultrathin sections were made on an Ultratoc-N ultramicrotome (Leica-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.

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 antero-posteriorly 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). Symbiote-like particles were observed in the cytoplast of the central and peripheral bacteriocytes (data not shown). The same configuration of the symbiotic organs was observed in D. corpulenta.
Endosymbionts of Drosicha Giant Scales

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 16S A1 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. 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 femniscata (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
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 (Uziniura 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.
Endosymbionts of Drosicha Giant Scales

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 synctial 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).

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, 1388 aligned nucleotide sites were analyzed. 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. E-symbiont, enterobacterial symbiont.

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).
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 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
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.

Gene analyses of the sharpshooter endosymbionts have suggested that the flavobacterial primary symbiont, Sulcia mulleri, provides the host with essential amino acids where

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.

<table>
<thead>
<tr>
<th>Lineage 1</th>
<th>Lineage 2</th>
<th>Outgroup</th>
<th>K1</th>
<th>K2</th>
<th>Difference in distance</th>
<th>Rate ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-symbionts of Drosicha scales</td>
<td>Free-living flavobacteria</td>
<td>Cytophaga hutchinsonii</td>
<td>0.104</td>
<td>0.103</td>
<td>0.001</td>
<td>1.01</td>
<td>0.95</td>
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<tr>
<td>E-symbionts of Drosicha scales</td>
<td>Free-living γ-proteobacteria</td>
<td>Yersinia pestis</td>
<td>0.015</td>
<td>0.023</td>
<td>−0.008</td>
<td>0.65</td>
<td>0.11</td>
</tr>
</tbody>
</table>

1 Estimated mean distance between lineages 1 and 2.
2 Estimated mean distance between lineages 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).
6 Kimura's two-parameter model (Kimura, 1980) was used to correct for multiple substitutions.
7 Genbank AE013602.
8 Genbank NC_008255.
9 Enterobacterial symbionts from Drosicha corpulenta (Tsukuba) [AB491196], D. corpulenta (Ueno) [AB491197], D. corpulenta (Ueno) [Uzou] [AB491198], and D. pinicola (Tsukuba) [AB491203].
10 Estimated mean distance between lineage 2 and the last common ancestor of lineages 1 and 2.
11 Genbank AE013602.

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