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Bacterial Symbionts of the Giant Jewel Stinkbug *Eucorysses grandis* (Hemiptera: Scutelleridae)

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Microbiological characterization of gut symbiotic bacteria in a limited number of stinkbugs of the families Acanthosomatidae, Plataspidae, Pentatomidae, Scutelleridae, Parastrachiidae, Alydidae and Pyrrhocoridae has shown symbiotic association with midgut bacteria to be common in phytophagous taxa of these heteropteran insects. Here we investigated the midgut bacterial symbiont of *Eucorysses grandis*, a stinkbug of the family Scutelleridae. A specific gammaproteobacterium was consistently identified in insects from five different geographic origins. The bacterium was detected in 64 of 64 insects sampled from three host populations. Phylogenetic analyses revealed that the bacterium constitutes a distinct lineage in the *Gammaproteobacteria*, neither closely related to the gut symbiont of another scutellerid stinkbug, *Cantao ocellatus*, nor to gut symbionts of other stinkbugs. Diagnostic PCR, in situ hybridization and electron microscopy demonstrated that the bacterium is located extracellularly, in the midgut fourth section, which possesses crypts. These results indicate that the primary gut symbionts have multiple evolutionary origins in the Scutelleridae. A *Sodalis*-allied facultative symbiont was also identified in some insects from natural populations. Biological aspects of the primary gut symbiont and the secondary *Sodalis*-allied symbiont are discussed.

Key words: *Eucorysses grandis*, Scutelleridae, gut symbiont, *Sodalis*, *Gammaproteobacteria*

INTRODUCTION

In the insect order Hemiptera, members of the suborder Heteroptera, commonly known as true bugs or stinkbugs, represent over 38,000 described species characterized by sucking mouthparts, half-membranous forewings, and incomplete metamorphosis (Schuh and Slater, 1995). In the Heteroptera, symbiotic associations with bacteria are common in plant-sucking taxa, particularly in stinkbugs of the infraorder Pentatomomorpha. These symbiotic species possess many sacs or tubular outgrowths, called crypts or caeca, in a posterior region of the midgut, wherein specific bacteria are harbored (Glasgow, 1914; Buchner, 1965). Aposymbiosis often results in retarded growth and high mortality in the insects (Abe et al., 1995; Fukatsu and Hosokawa, 2002; Hosokawa et al., 2006; Kikuchi et al., 2007; Kikuchi et al., 2009; Prado and Almeida, 2009b), indicating that the symbionts play a physiological role for the hosts. The symbionts are vertically transmitted across host generations by several post-hatch transmission mechanisms: (i) egg surface contamination in the Pentatomidae, Acanthosomatidae and Scutelleridae (Rosenkranz, 1939; Abe et al., 1995; Prado et al., 2006; Kikuchi et al., 2009; Prado and Almeida, 2009a; Kaiwa et al., 2010), (ii) coprophagy in the Cydnidae and Coreidae (Huber-Schneider, 1957; Schorr, 1957), or (iii) capsule transmission in the Plataspidae (Hosokawa et al., 2005), whereas a case of environmental acquisition has been reported from the Alydidae (Kikuchi et al., 2007). To date, the gut symbiotic bacteria of a limited number of the stinkbug families Acanthosomatidae, Plataspidae, Pentatomidae, Scutelleridae, Parastrachiidae, Alydidae and Pyrrhocoridae have been subjected to modern microbiological characterization (Kikuchi et al., 2005, 2009; Hosokawa et al., 2006, 2010; Kaltenpoth et al., 2009; Prado and Almeida, 2009a; Kaiwa et al., 2010).

Members of the family Scutelleridae, often referred to as jewel bugs or shield-backed bugs, are known for their vivid and beautiful body coloration (Schuh and Slater, 1995). The family includes some 80 genera and 450 species, and in Japan, at least seven genera and nine species have been recorded (Tomokuni et al., 1993). Early histological works described the presence of symbiotic bacteria in midgut

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crypts of several scutellerid species (Glasgow, 1914; Kuskop, 1923; Rosenkranz, 1939). Since that time, however, the microbial nature of scutellerid symbionts has remained obscure. Recently, a gammaproteobacterial primary gut symbiont and a *Sodalis*-allied facultative symbiont were identified from the scutellerid stinkbug *Cantao ocellatus* (Kaiwa et al., 2010). However, it is uncertain whether these bacterial symbionts are commonly found in other scutellerid species.

In this study, we investigated the endosymbiotic microbiota of another scutellerid stinkbug, *Eucorysses grandis* (Fig. 1A). A gammaproteobacterial gut symbiont and a *Sodalis*-allied facultative symbiont were identified in *E. grandis*, as in *C. ocellatus*, but the gut symbiont was phylogenetically distinct from that of *C. ocellatus*, suggesting multiple evolutionary origins of the primary gut symbionts in the Scutelleridae.

MATERIALS AND METHODS

Insect materials

Table 1 shows collection data of *E. grandis* used in this study. Adult insects were collected at five localities in areas of southern Japan.

Dissection and DNA extraction

The first, second, third, and fourth sections of midgut, as well as ovaries and testes, were dissected from adult insects using a pair of fine forceps under a dissection microscope in a glass Petri dish filled with phosphate buffered saline (PBS; 137 mM NaCl/8.1 mM Na_2HPO_4/2.7 mM KCl/1.5 mM KH_2PO_4 [pH 7.5]). The dissected tissues were either immediately subjected to DNA extraction using Nucleo Spin® Tissue kit (Macherey-Nagel), or preserved in acetone until use (Fukatsu, 1999).

DNA cloning and sequencing

A 1.5 kb segment of bacterial 16S rRNA gene was amplified with the primers 16SA1 (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 16SB1 (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) (Fukatsu and Nikoh, 1998). Polymerase chain reaction (PCR) was conducted with AmpliTaq Gold*Taq* DNA polymerase (Applied Biosystems) and the supplemented buffer system, following a temperature profile of 95°C for 10 min, followed by 35 cycles consisting of 95°C for 30 sec, 55°C for 1 min, and 72°C for 2 min. Cloning and sequencing of the amplified products were performed as previously described (Kaiwa et al., 2010).

Diagnostic PCR

The following specific primer sets were used for diagnostic PCR detection of 16S rRNA gene of the symbiotic bacteria: ookin162F (5′-GCT AAT ACC GCA TAA CGT CTT-3′) and

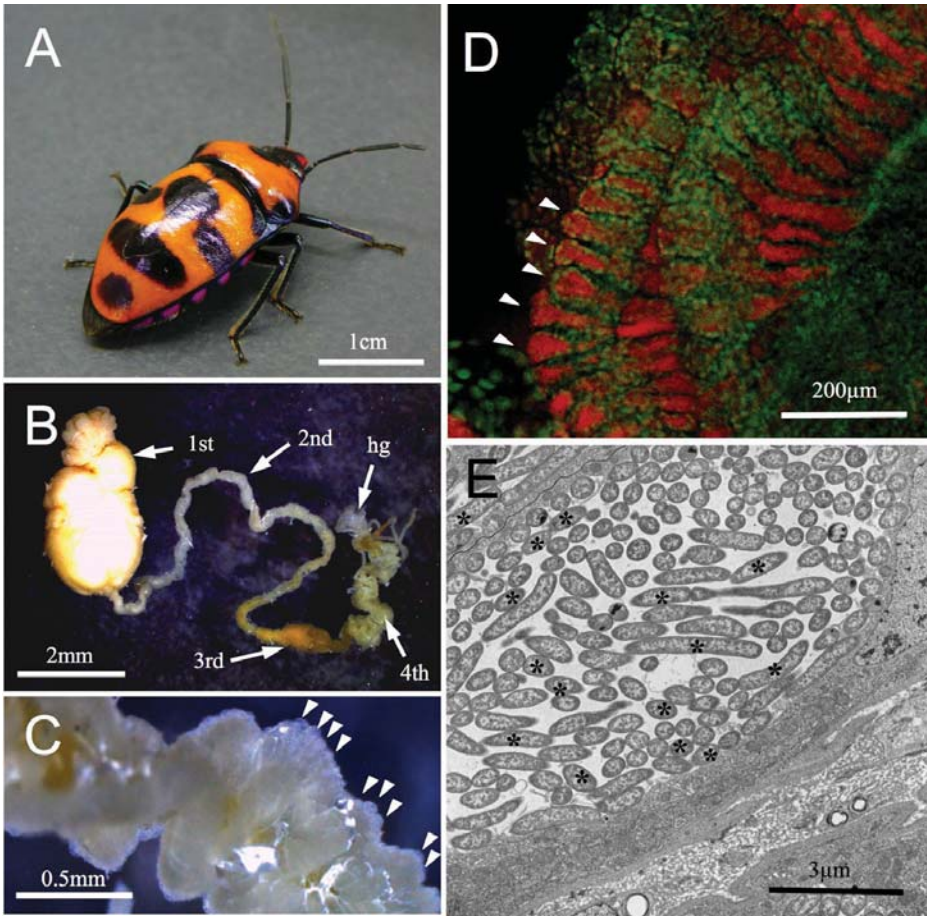

Fig. 1. **(A)** An adult female of *E. grandis*. **(B)** A dissected midgut from an adult female of *E. grandis*. 1st, midgut first section; 2nd, midgut second section; 3rd, midgut third section; fourth, midgut 4th section with crypts; hg, hindgut. **(C)** An enlarged image of the midgut fourth section with crypts. Arrowheads indicate rows of crypts. **(D)** Detection of bacterial 16S rRNA in the midgut fourth section by in situ hybridization. **(E)** A transmission electron microscopic image of a midgut crypt, whose cavity is full of bacterial cells. Asterisks indicate the symbiont cells.

Table 1. Samples of *Eucorysses grandis* used in this study.

Collection locality	Collection date	Collector	Host plant	Accession number	
				Gut symbiont	*Sodalis* symbiont
Yakushima, Kagoshima, Japan	November 2008	Takema Fukatsu	*Schefflera octophylla*	AB571334	AB571330
Nishino-Omote, Kagoshima, Japan	July 2009	Takahiro Hosokawa	*Vernicia cordata*	AB571333	
Kashima, Saga, Japan	September 2009	Fumitaka Kuchiki	Unknown	AB571332	
Minami-Boso, Chiba, Japan	March 2009	Takahiro Hosokawa	*Pittosporum tobira*	AB571331	
Tosa-Shimizu, Kochi, Japan	November 2001	Takuya Takemoto	Unknown	AB571335	

ookin616R (5′-TCT ACG AGA CTC AAG CCT GT-3′) for specific detection of a 0.45 kb segment from the gut symbiont; and sodalis370F (5′-CGR TRG CGT TAA YAG CGC-3′) and 16SB2 (5′-CGA GCT GAC GAC ARC CAT GC-3′)(Kaiwa et al., 2010) for specific detection of a 0.62 kb segment from the *Sodalis*-allied symbiont. The PCR temperature profile was 95°C for 10 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. To confirm the quality of the DNA samples, a 1.5 kb segment of the insect mitochondrial 16S rRNA gene was amplified with the primers MtrA1 (5′-AAW AAA CTA GGA TTA GAT ACC CTA-3′) and MtrB1 (5′-TCT TAA TYC AAC ATC GAG GTC GCA A-3′) (Fukatsu et al., 2001).

In situ hybridization

An Alexa555-labeled oligonucleotide probe EUB338 (5′-GCT GCC TCC CGT AGG AGT-3′) (Amann et al., 1990) was used for whole mount fluorescent in situ hybridization targeting eubacterial 16S rRNA. Midgut preparations with crypts were dissected from adult females and fixed in Carnoy's solution (ethanol: chloroform: acetic acid = 6: 3: 1) overnight. The fixed samples were incubated in ethanolic 6% H_2O_2 solution for a week to quench the autofluorescence of the tissues (Koga et al., 2009). The samples were thoroughly washed and equilibrated with a hybridization buffer (20 mM Tris HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 30% formamide), to which the probe and SYTOX green were added at final concentrations of 0.1 μM and 0.5 μM, respectively. After overnight incubation, the samples were thoroughly washed in PBSTx (PBS containing 0.3% Triton X-100) and observed under an epifluorescence microscope (Axiophoto, Carl Zeiss) and a laser confocal microscope (PASCAL5, Carl Zeiss).

Electron microscopy

Midgut crypts were dissected from adult females in 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde, prefixed in the fixative at 4°C overnight, and postfixed in the phosphate buffer containing 2% osmium tetroxide at 4°C for 60 min. After dehydration through an ethanol series, the materials were embedded in Spurr resin (Nisshin-EM). Ultrathin sections (thickness, 80 nm) were made on an ultramicrotome (Ultracat-N, Leichert-Nissei), mounted on collodion-coated copper meshes, stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (model H-7000, Hitachi).

Molecular phylogenetic and evolutionary analyses

A multiple alignment of nucleotide sequences was generated using the program Clustal W (Thompson et al., 1994), and the final alignment was corrected manually. Phylogenetic analyses were conducted by maximum likelihood and maximum parsimony methods. The best-fit substitution model was selected by using the program MrModeltest v2.1 (J. A. Nylander, 2004; http://www.ebc.uu.se/systzoo/staff/nylander.html). A maximum likelihood phylogeny, with 100 bootstrap resamplings, was estimated using the program phyML 3.0 (Guindon et al., 2005) under the GTR model. A maximum parsimony phylogeny, with 1,000 bootstrap resamplings, was estimated using the program phylo_win (Galtier, 1996). A relative rate test, based on genetic distances estimated under the Kimura's two-

Table 2. Infection frequencies of the gut symbiont and the *Sodalis*-allied symbiont in natural populations of *Eucorysses grandis*.

Population[1]	Gut symbiont[2]			*Sodalis*-allied symbiont[3]		
	Male	Female	Total	Male	Female	Total
Yakushima	100%	100%	100%	13%	13%	13%
	(15/15)	(15/15)	(30/30)	(2/15)	(2/15)	(4/30)
Kashima	100%	100%	100%	0%	0%	0%
	(2/2)	(10/10)	(12/12)	(0/2)	(0/10)	(0/12)
Minami-Boso	100%	100%	100%	0%	0%	0%
	(10/10)	(12/12)	(22/22)	(0/10)	(0/12)	(0/22)
Total	100%	100%	100%	7%	5%	6%
	(27/27)	(37/37)	(64/64)	(2/27)	(2/37)	(4/64)

[1]Nishino-Omote and Tosa-Shimizu populations were excluded because only a few insects were obtained from each population.
[2]DNA samples from dissected midgut were analyzed.
[3]DNA samples from dissected gonads were analyzed.

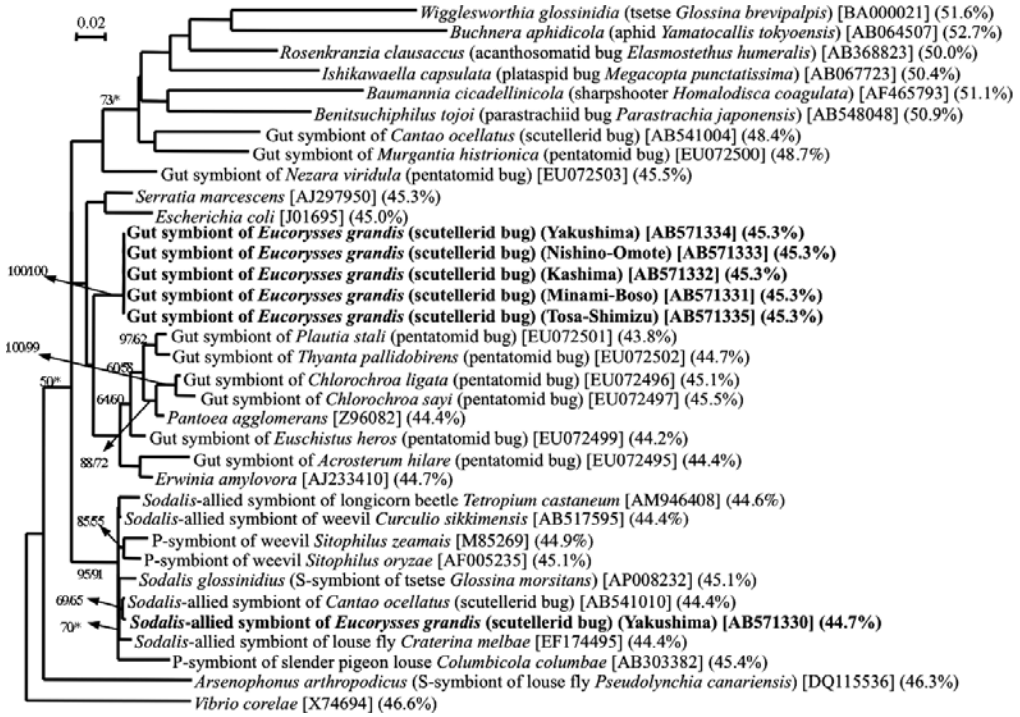

Fig. 2. Phylogenetic placement of the symbiotic bacteria from *E. grandis* on the basis of 16S rRNA gene sequences. A maximum likelihood (ML) tree inferred from a total of 1,005 unambiguously aligned nucleotide sites is shown, whereas a maximum parsimony (MP) analysis gave substantially the same topology (data not shown). Bootstrap values higher than 50% are indicated at the nodes in the order ML/MP. Asterisks indicate support values lower than 50%. Sequence accession numbers and AT contents of the nucleotide sequences are in brackets and parentheses, respectively. As for insect endosymbionts, the name of the host insect is also indicated in parentheses. P-symbiont, primary symbiont; S-symbiont, secondary symbiont.

Table 3. Relative-rate test for comparing the molecular evolutionary rates of 16S rRNA gene sequences between the lineages of the gut symbiont and the *Sodalis*-allied symbiont of *Eucorysses grandis,* and their free-living relatives.

Lineage 1	Lineage 2	Outgroup	K1[1]	K2[2]	K1 – K2	K1/K2	*P*-value[3]
Gut symbiont of *E. grandis* [AB571334]	*Escherichia coli* [J01695]	*Vibrio cholerae* [X74694]	0.018	0.025	–0.007	0.72	0.253
Sodalis-allied symbiont of *E. grandis* [AB571330]	*Escherichia coli* [J01695]	*Vibrio cholerae* [X74694]	0.027	0.028	–0.001	0.96	0.826

[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]*P*-value was generated using the program RRTree (Robinson-Rechavi and Huchon, 2000).

parameter model (Kimura, 1980), was performed using the program RRTree (Robinson-Rechavi and Huchon, 2000).

RESULTS

Detection of bacterial symbiont in the midgut fourth section of *E. grandis*

Digestive organs were dissected from adult females of *E. grandis* (Fig. 1B). The midgut first section was determined to be a large stomach-like structure filled with liquid, followed by tubular midgut second and third sections. The midgut fourth section was equipped with a number of crypts, which were white in color, arranged in four rows, and fused to each other into a loose helical assemblage (Fig. 1C). In situ hybridization detected strong bacterial signals in the crypts of the midgut fourth section (Fig. 1D). Electron microscopy identified tubular bacterial cells of a single morphotype densely packed in the cavity of the midgut crypts (Fig. 1E). A 16S rRNA gene sequence (1,465 bp) was cloned and determined from the midgut fourth section of a female insect of Yakushima population, to which the sequences obtained from insects of Minami-Boso, Tosa-Shimizu, Kashima and Nishino-Omote populations were identical. BLAST searches using this sequence as the query retrieved gammaproteobacterial sequences, of which the top hit was a 16S rRNA gene sequence of uncultured bacterium isolated from mucosal biopsy from human intestinal pouch (98.5% [1,443/1,465] identity; accession number GQ157226).

Detection of another bacterial symbiont in the gonads of *E. grandis*

A bacterial 16S rRNA gene was detected by PCR on ovarian DNA of a female insect from the Yakushima population. Cloning and sequencing of the PCR product yielded a 1,464 bp sequence. BLAST searches using this sequence as the query retrieved gammaproteobacterial sequences, of which the top hit was 16S rRNA gene sequence of the *Sodalis*-allied symbiont of *Cantao ocellatus* (Hemiptera; Scutelleridae) (99.7% [1,459/1,464] identity; accession number AB541010).

Prevalence of the bacterial symbionts in natural populations of *E. grandis*

Diagnostic PCR surveys showed the prevalence of the gut symbiont in natural populations of *E. grandis*; of 64 insects from three populations examined, all were PCR-positive. By contrast, the *Sodalis*-allied symbiont was detected in only a subset of the insects: 13% (4/30), 0% (0/12) and 0% (0/22) in Yakushima, Kashima and Minami-Boso populations, respectively (Table 2).

Phylogenetic placement of the bacterial symbionts from *E. grandis*

Figure 2 shows the phylogenetic placement of the bacterial symbionts detected from *E. grandis* on the basis of 16S rRNA gene sequences. None of the gut symbionts of other stinkbugs, including that of the scutellerid *C. ocellatus* (Kaiwa et al., 2010), was related to the gut symbiont of *E. grandis*. Meanwhile, the *Sodalis*-allied symbionts of *E. grandis* and *C. ocellatus* formed a well-supported gammaproteobacterial clade with the secondary symbiont of the tsetse fly *Sodalis glossinidius*, the bacteriome-associated symbiont of grain weevils, the symbiont of a louse fly, the bacteriocyte-associated symbiont of a pigeon louse, and a secondary symbiont of a chestnut weevil.

Molecular evolutionary aspects of the bacterial symbionts from *E. grandis*

The 16S rRNA sequences of the gut symbiont and the *Sodalis*-allied symbiont exhibited AT contents of 45.3% and 44.7%, respectively, which were not different from AT contents of other phylogenetically-related free-living gammaproteobacteria at around 45% (Fig. 2). The evolutionary rates of the 16S rRNA gene sequences in the gut symbiont and the *Sodalis*-allied symbiont were not significantly different from the evolutionary rates in phylogenetically-related free-living gammaproteobacteria (Table 3).

DISCUSSION

We demonstrated that *E. grandis* harbors a specific gammaproteobacterial symbiont in the cavity of the midgut crypts (Fig. 1; Fig. 2). The 100% infection frequencies in natural populations (Table 2) suggest that the gut symbiont is the primary obligate symbiont of *E. grandis*, although the biological role of the symbiont for the host, if any, is unknown. In addition to the gut symbiont, a *Sodalis*-allied symbiont was identified from gonad of *E. grandis*. The partial infection frequencies in natural populations (Table 2) indicate that the *Sodalis*-allied symbiont is a secondary, facultative symbiont of *E. grandis*.

A recent study reported that the scutellerid stinkbug *C. ocellatus* possesses a gammaproteobacterial primary gut symbiont and a *Sodalis*-allied secondary symbiont (Kaiwa et al., 2010). This symbiotic configuration may appear similar to that of *E. grandis*. However, molecular phylogenetic analysis clearly showed that the gut symbiont of *E. grandis* is phylogenetically distinct from that of *C. ocellatus* (Fig. 2), indicating that the primary gut symbionts of scutellerid stink-

bugs are not monophyletic, but of multiple evolutionary origins.

Previous studies have identified gammaproteobacterial midgut symbionts from diverse stinkbugs of the pentatomomorphan families Acanthosomatidae, Plataspidae, and Pentatomidae. Although these symbionts are commonly localized in the same symbiotic organ, the midgut fourth section with crypts, their microbiological affiliations are different: *Rosenkranzia clausaccus* in the Acanthosomatidae (Kikuchi et al., 2009), *Ishikawaella capsulata* in the Plataspidae (Hosokawa et al., 2006), and unnamed gammaproteobacterial lineages in the Pentatomidae (Prado and Almeida, 2009a). In the Acanthosomatidae and Plataspidae, the gut symbionts are respectively monophyletic and cospeciate with the host insects, reflecting strict host-symbiont co-evolution over evolutionary time (Hosokawa et al., 2006; Kikuchi et al., 2009). By contrast, the gut symbionts are polyphyletic in the Pentatomidae, suggesting occasional lateral transfers and/or replacements of the symbionts (Prado and Almeida, 2009a). The situation in the Scutelleridae turned out to be similar to the situation in the Pentatomidae, and not to that in the Acanthosomatidae/Plataspidae.

With limited information on only two species, *C. ocellatus* and *E. grandis*, the diversity of the gut symbionts in the Scutelleridae warrants future study. It should be noted that the gut symbiont of *C. ocellatus* exhibits AT-biased nucleotide composition and accelerated molecular evolution (Kaiwa et al., 2010), whereas the gut symbiont of *E. grandis* does not (Fig. 2; Table 3). Given that these peculiar genetic traits are often associated with long-lasting intimate symbiotic associations (Wernegreen, 2002), it may be that the evolutionary origin of the gut symbiont of *E. grandis* is more recent than that of *C. ocellatus*.

It is notable that, although symbiotic bacteria have been examined in only two scutellerid stinkbugs, *Sodalis*-allied secondary symbionts were identified in both cases. For a long time, *Sodalis glossinidius* and allied symbionts have been known only from tsetse flies and grain weevils (Dale and Maudlin, 1999; Heddi and Nardon, 2005). However, recent studies have uncovered an unexpected prevalence of *Sodalis*-allied symbionts among diverse insects including a louse fly (Novakova and Hypsa, 2007), a pigeon louse (Fukatsu et al., 2007), a chestnut weevil (Toju et al., 2010), and a longicorn beetle (Grünwald et al., 2010). Infection prevalence and biological roles of the *Sodalis*-allied symbionts in scutellerid and other stinkbugs are of interest for future study. The scutellerid stinkbugs are unique in that the obligate primary symbiont is extracellular in the midgut cavity, whereas the facultative secondary symbiont is associated with the host gonads. The primary extracellular symbiont and the secondary endosymbiont coexisting in the same host body may unveil a previously unknown aspect of microbe-microbe interactions in insect symbiosis.

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