ORIGINAL ARTICLE

An ancient but promiscuous host–symbiont association between *Burkholderia* gut symbionts and their heteropteran hosts

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Here, we investigated 124 stinkbug species representing 20 families and 5 superfamilies for their *Burkholderia* gut symbionts, of which 39 species representing 6 families of the superfamilies Lygaeoidea and Coreoidea were *Burkholderia*-positive. Diagnostic PCR surveys revealed high frequencies of *Burkholderia* infection in natural populations of the stinkbugs, and substantial absence of vertical transmission of *Burkholderia* infection to their eggs. *In situ* hybridization confirmed localization of the *Burkholderia* in their midgut crypts. In the lygaeoid and coreoid stinkbugs, development of midgut crypts in their alimentary tract was coincident with the *Burkholderia* infection, suggesting that the specialized morphological configuration is pivotal for establishment and maintenance of the symbiotic association. The *Burkholderia* symbionts were easily isolated as pure culture on standard microbiological media, indicating the ability of the gut symbionts to survive outside the host insects. Molecular phylogenetic analysis showed that the gut symbionts of the lygaeoid and coreoid stinkbugs belong to a β-proteobacterial clade together with *Burkholderia* isolates from soil environments and *Burkholderia* species that induce plant galls. On the phylogeny, the stinkbug-associated, environmental and gall-forming *Burkholderia* strains did not form coherent groups, indicating host–symbiont promiscuity among these stinkbugs. Symbiont culturing revealed that slightly different *Burkholderia* genotypes often coexist in the same insects, which is also suggestive of host–symbiont promiscuity. All these results strongly suggest an ancient but promiscuous host–symbiont relationship between the lygaeoid/coreoid stinkbugs and the *Burkholderia* gut symbionts. Possible mechanisms as to how the environmentally transmitted promiscuous symbiotic association has been stably maintained in the evolutionary course are discussed.

Introduction

Many insects possess symbiotic microorganisms of mutualistic nature either in the gut lumen, in the body cavity or inside the cells. In general, such microbial partners are maintained through host generations by specialized transmission mechanisms (Buchner, 1965; Bourtzis and Miller, 2003; Kikuchi, 2009; Bright and Bulgheresi, 2010). For example, an intracellular symbiont *Buchnera* in aphids is providing essential amino acids for the host growth and

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reproduction, and transovarially transmitted to eggs or embryos in the maternal host body (Douglas, 1998; Miura *et al.*, 2003). Meanwhile, insects harboring extracellular gut symbionts of mutualistic nature, like termites and stinkbugs, have evolved posthatch mechanisms for symbiont transmission, such as superficial microbial contamination of eggs (egg smearing) and probing of parental microbe-containing excrement (coprophagy) (reviewed in Buchner, 1965). *In utero* symbiont transmission via milk gland secretion in tsetse flies (Attardo *et al.*, 2008) and capsule-mediated symbiont transmission in plataspid stinkbugs (Hosokawa *et al.*, 2005) represent unique posthatch transmission mechanisms. In these and other diverse insects, the mechanisms for symbiont transmission thus far described are mostly vertical ones from parents to offspring.

On the other hand, environmental symbiont transmission, wherein newborn host organisms acquire

their mutualistic partners from environmental microbial pool every generation, has been known from diverse marine invertebrates including luminescent squids (Nyholm and McFall-Ngai, 2004), photosynthetic corals (Baker, 2003), chemoautotrophic tubeworms (Dubilier *et al.*, 2008) and many others. In the aquatic ecosystem, free-living symbionts are protected from environmental stresses such as UV irradiation and desiccation that must be detrimental to free-living symbionts in the terrestrial ecosystem, to which the paucity of environmental symbiont transmission among insects may be relevant. Even in the terrestrial ecosystem, however, some plants are mutualistically associated with specific soil microbes, as in legume–*Rhizobium* and alder–*Frankia* nitrogen-fixing symbioses, wherein environmental symbiont transmission occurs (Denarie *et al.*, 1992; Benson and Silvester, 1993). It should be noted that in these cases, the symbionts outside the host organisms are also protected from the above-mentioned environmental stresses in the soil. In this context, it is remarkable as well as understandable that environmental symbiont transmission was recently discovered in an insect–microbe mutualism, wherein the symbiont was soil bacteria of the genus *Burkholderia* (Kikuchi *et al.*, 2007).

Members of the suborder Heteroptera, known as true bugs or stinkbugs, comprise a large group of insects with incomplete metamorphosis. The infraorder Pentatomomorpha, placed under the Heteroptera, embraces over 12 500 species that are grouped into five superfamilies (Figure 1; Schaefer, 1993; Schuh and Slater, 1995; Henry, 1997). Besides relatively minor predacious and mycophagous species, the majority of pentatomomorphan stinkbugs are plant sap feeders, some of which are notorious as agricultural pests (Schaefer and Panizzi, 2000). In most of the phytophagous species, a number of sac- or tube-like outgrowths (called crypts or caeca) develop in a posterior region of the midgut (so-called midgut fourth section), wherein specific bacteria are harbored (Glasgow, 1914; Miyamoto, 1961; Buchner, 1965; Kikuchi *et al.*, 2008). In the families Pentatomidae, Plataspidae, Acanthosomatidae, Scutelleridae and Parastrachiidae, their gut symbionts belong to distinct lineages in the γ-*Proteobacteia*, indicating multiple evolutionary origins of the symbiotic associations (Fukatsu and Hosokawa, 2002; Hosokawa *et al.*, 2006, 2010; Prado *et al.*, 2006; Kikuchi *et al.*, 2009; Prado and Almeida, 2009a, b; Kaiwa *et al.*, 2010). In several species of the families Pentatomidae, Plataspidae, Acanthosomatidae, Parastrachiidae and Cydnidae, symbiont-free insects suffered retarded growth and/or nymphal mortality, suggesting mutualistic nature of the symbiotic associations (Müller, 1956; Huber-Schneider, 1957; Schorr, 1957; Abe *et al.*, 1995; Fukatsu and Hosokawa, 2002; Hosokawa *et al.*, 2006; Kashima *et al.*, 2006; Kikuchi *et al.*, 2009; Prado and Almeida, 2009b).
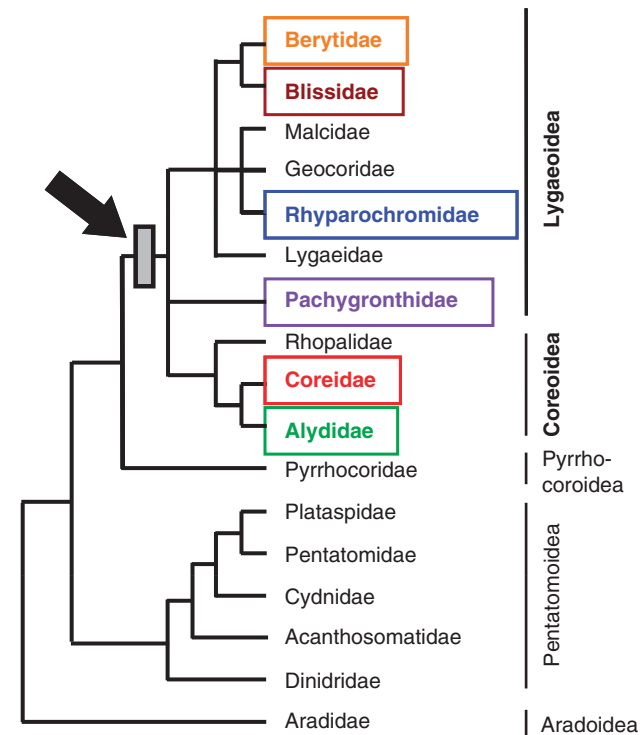

Figure 1 Phylogenetic relationship of the pentatomomorphan stinkbug families. The tree is based on the nuclear 18S rRNA gene phylogeny by Xie *et al.* (2005) and the mitochondrial whole-genome phylogeny by Hua *et al.* (2008). Both studies strongly supported the phylogenetic relationship (Aradoidea + (Pentatomoidea + (Pyrrhocoroidea + (Coreoidea + Lygaeoidea)))). Names of stinkbug superfamilies are depicted on the right side. Names of stinkbug families associated with the *Burkholderia* symbionts are shown in colors. An arrow indicates the hypothetical evolutionary origin of the stinkbug–*Burkholderia* association.

All these gut symbionts are vertically transmitted through host generations either via egg smearing in the Pentatomidae, Acanthosomatidae and Scutelleridae (Abe *et al.*, 1995; Prado *et al.*, 2006; Kikuchi *et al.*, 2009; Prado and Almeida, 2009b; Kaiwa *et al.*, 2010), via coprophagy in the Cydnidae (Schorr, 1957), or via capsule transmission in the Plataspidae (Hosokawa *et al.*, 2005).

The bean bug *Riptortus pedestris* (or *Riptortus clavatus* as synonym) is known as pest of leguminous crops in eastern Asia (Schaefer and Panizzi, 2000). Whereas the above-mentioned stinkbug families belong to the superfamily Pentatomoidea, *R. pedestris* is a member of the family Alydidae in the superfamily Coreoidea (see Figure 1). Similar to the pentatomoid stinkbugs, *R. pedestris* possesses numerous crypts in the posterior midgut, but the bacterium in the symbiotic organ is quite different, belonging to the genus *Burkholderia* in the β-*Proteobacteria* (Kikuchi *et al.*, 2005). Aposymbiotic insects suffered marked reduction in body size and weight, indicating mutualistic nature of the symbiosis (Kikuchi *et al.*, 2007). However, distinct from the pentatomoid symbionts thus far studied, the *Burkholderia* symbiont was present in the rhizosphere of the food plants of *R. pedestris*, easily

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culturable on standard microbiological media, and orally acquired by young nymphs of *R. pedestris* from the environment without vertical transmission (Kikuchi *et al.*, 2007). The discovery of environmental acquisition of beneficial symbiont without vertical transmission in *R. pedestris* was unprecedented among insects. As the *Burkholderia* gut symbiont was also detected in a related alydid species *Leptocorisa chinensis* (Kikuchi *et al.*, 2005), the case of *R. pedestris* cannot be an orphan exception. It is of interest whether or not such symbiotic associations are also found in other stinkbug species.

In this study, therefore, we surveyed 124 pentatomomorphan stinkbugs representing 20 families and 5 superfamilies for infection with the *Burkholderia* symbionts, identified 39 *Burkholderia*-associated stinkbugs from 6 families and 2 superfamilies, and investigated their phylogenetic relationship, localization *in vivo*, transmission mode and culturability *in vitro*.

Materials and methods

Insect materials
Samples of Japanese pentatomomorphan species examined in this study are listed in Table 1 and Supplementary Table S1. These insects were collected in 1995–2007 and preserved in acetone at room temperature until use (Fukatsu, 1999).

DNA extraction
Acetone-preserved adult insects were individually dissected in a phosphate-buffered saline (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mMKH_2PO_4 (pH 7.5)), from which the midgut fourth section with crypts was collected. For the stinkbug species that possess no midgut crypts, the whole area of the midgut was collected. The dissected tissues were homogenized in 200 μl of lysis buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 M NaCl, 0.5% SDS, 0.2 mg ml^{-1} proteinase K) and incubated at 56 °C for 2 h. The lysate was extracted with 200 μl of phenol:chloroform:isoamyl alcohol (25:24:1). DNA was recovered from the purified lysate by ethanol precipitation, and dissolved in 200 μl of TE buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA).

Diagnostic PCR
A 0.75 kb region of 16S rRNA gene of *Burkholderia* spp. was amplified by the specific primers Burk16SF (5′-TTTTGGACAATGGGGGCAAC-3′) and Burk16SR (5′-GCTCTTGCGTAGCAACTAAG-3′) under a temperature profile of 95 °C for 10 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min (Kikuchi *et al.*, 2005). To check the quality of template DNA samples, a 0.65 kbp region of insect mitochondrial *cytochrome oxidase I* gene was amplified with the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994).

Cloning, genotyping and sequencing
A 1.5 kbp region of bacterial 16S rRNA gene was amplified by PCR with the universal primers 16SA1 and 16SB1 under a temperature profile of 95 °C for 10 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min (Fukatsu and Nikoh, 1998). The PCR products were cloned with a TA-cloning vector pT7Blue (Takara) and *Escherichia coli* DH5α competent cells (Takara). To check the length of the inserted DNA fragment, white colonies expected to contain inserted plasmid were directly subjected to PCR with the primers Univ19 (5′-GT TTTCCCAGTCACGACGT-3′) and Rev20 (5′-AGCTA TGACCATGATTACGC-3′). When a PCR product of expected size (1.5 kbp) was obtained, the product was digested by restriction endonucleases *Hae*III and *Rsa*I, and electrophoresed in agarose gels. Three clones or more from each of all restriction genotypes were cultured and subjected to plasmid extraction using QIAprep-Spin Miniprep Kit (QIAGEN, Chuo-ku, Japan). The purified plasmids were eluted with 50 μl of distilled water and subjected to DNA sequencing with DNA Sequencing Kit FS (PerkinElmer, Chiyoda-ku, Japan) and 3130xl Genetic Analyzer (Applied Biosystems, Chuo-ku, Japan).

Molecular phylogenetic analysis
Multiple alignments of the nucleotide sequences were generated by the program Clustal W (Thompson *et al.*, 1994) and then manually realigned using the program SeaView (Galtier *et al.*, 1996). Neighbor-joining (NJ) trees were constructed using the Clustal W under the Kimura's two-parameter model (Kimura, 1980). Maximum likelihood (ML) trees were estimated by the program Phylo_Win (Galtier *et al.*, 1996) under the HKY substitution model (Hasegawa *et al.*, 1985). Bootstrap tests were performed with 1000 and 100 replications in the NJ and ML analyses, respectively.

In situ *hybridization*
Two oligonucleotide probes Alsym16S (5′-ACACTC AAAGCCTGCCAGT-3′; Kikuchi *et al.*, 2005) and BURK129 (5′-CCACTACAGGACACGTTC-3′), whose 5′ end was labeled with AlexaFluor555, were used for *in situ* hybridization. These probes specifically targeted different regions in 16S rRNA of the *Burkholderia* symbionts, and were simultaneously used for enhancement of the hybridization signals. The tissue samples were incubated in a hybridization buffer (20 mM Tris-HCl (pH 8.0), 0.9 M NaCl, 0.01% SDS, 30% formamide) containing 50 nM each of the probes and 0.5 μM of SYTOX Green (Invitrogen, Chuo-ku, Japan). After an overnight incubation, the samples were thoroughly washed in phosphate-buffered saline and mounted in Slow-fade antifade solution (Molecular Probes, Chuo-ku, Japan), and observed under an epifluorescent microscope (Axiophot, Carl Zeiss, Shinjuku-ku, Japan)

Table 1 Pentatomomorphan stinkbugs examined in this study[a]

Superfamily family	Species	Feeding type[b]	Crypt type[c]	Burkholderia infection[d]
Aradoidea				
Aradidae	*Neuroctenus castaneus*	Myco	—	—
Pyrrhocoroidea				
Pyrrhocoridae	*Antilochus coqueberti*	Pred	—	—
	Pyrrhocoris sibiricus	Phyto	—	—
	Pyrrhocoris sinuaticollis	Phyto	—	—
	Dysdercus cingulatus	Phyto	—	—
	Dysdercus poecilus	Phyto	—	—
	Dysdercus decussatus	Phyto	—	− (0/2)
Lygaeoidea				
Berytidae	*Yemma exilis*[e,f]	Phyto	Tubes	+ (10/11)
	Metatropis rufescens	Phyto	Tubes	+
Malcidae	*Chauliops fallax*	Phyto	—	—
	Malcus japonicus	Phyto	—	—
Lygaeidae	*Tropidothorax belogolowi*	Phyto	—	—
	Aethalotus nigriventris	Phyto	—	—
	Caenocoris dimidiatus	Phyto	—	—
	Spilostethus hospes	Phyto	—	− (0/4)
	Graptostethus servus	Phyto	—	—
	Oncopeltus nigriceps	Phyto	—	—
	Thunbergia sanguinaria	Phyto	—	—
	Nysius plebeius	Phyto	—	—
	Pylorgus colon	Phyto	—	—
Geocoridae	*Geocoris proteus*	Pred	—	—
	Piocoris varius	Pred	—	—
Blissidae	*Dimorphopterus pallipes*[e,f]	Phyto	Tubes	+ (31/31)
	Cavelerius saccharivorus	Phyto	Tubes	+
Rhyparochromidae	*Pachybrachius luridus*	Phyto	Tubes	+
	Paromius exiguus[e,f]	Phyto	Tubes	+
	Togo hemipterus[e,f]	Phyto	Tubes	+ (36/36)
	Paraeucosmetus pallicornis	Phyto	Tubes	+
	Horridipamera inconspicua	Phyto	Tubes	+
	Horridipamera nietneri	Phyto	Tubes	+
	Panaorus japonicus[e]	Phyto	Tubes	+
	Panaorus albomaculatus	Phyto	Tubes	+
	Metochus abbreviatus	Phyto	Tubes	+
	Neolethaeus dallasi	Phyto	2 Rows	+
	Lethaeus assamensis	Phyto	2 Rows	+
Pachygronthidae	*Pachygrontha antennata*[e,f]	Phyto	2 Rows	+ (18/18)
Coreoidea				
Coreidae	*Molipteryx fuliginosa*[e]	Phyto	2 Rows	+
	Acanthocoris sordidus[e,f]	Phyto	2 Rows	+ (3/3)
	Leptoglossus australis	Phyto	2 Rows	+
	Dasynus coccocinctus	Phyto	2 Rows	+
	Notobitus meleagris[e]	Phyto	2 Rows	+
	Hygia opaca[e]	Phyto	2 Rows	+
	Hygia lativentris[e,f]	Phyto	2 Rows	+ (3/3)
	Homoeocerus dilatatus[e]	Phyto	2 Rows	+
	Homoeocerus unipunctatus	Phyto	2 Rows	+ (3/3)
	Homoeocerus marginiventris	Phyto	2 Rows	+
	Anacanthocoris striicornis	Phyto	2 Rows	+
	Paradasynus spinosus	Phyto	2 Rows	+
	Plinachtus bicoloripes[e]	Phyto	2 Rows	+
	Plinachtus basalis	Phyto	2 Rows	+
	Cletus trigonus[e]	Phyto	2 Rows	+
	Cletus punctiger[e,f]	Phyto	2 Rows	+ (22/25)
	Cletus rusticus[e]	Phyto	2 Rows	+
Alydidae	*Riptortus pedestris*[f]	Phyto	2 Rows	+
	Riptortus linearis[e]	Phyto	2 Rows	+
	Leptocorisa chinensis[f]	Phyto	2 Rows	+
	Leptocorisa acuta	Phyto	2 Rows	+
	Leptocorisa oratoria	Phyto	2 Rows	+
	Megalotomus costalis	Phyto	2 Rows	+
	Daclera levana[e]	Phyto	2 Rows	+
	Paraplesius unicolor	Phyto	2 Rows	+
Rhopalidae	*Leptocoris rufomarginata*	Phyto	—	—
	Rhopalus maculates	Phyto	—	− (0/4)
	Stictopleurus punctatonervosus	Phyto	—	− (0/6)
Pentatomoidea				
Urostylidae	*Urochela luteovaria*	Phyto	4 Rows	—
	Urostylis annulicornis	Phyto	4 Rows	—

Table 1 (Continued)

Superfamily family	Species	Feeding type[b]	Crypt type[c]	Burkholderia infection[d]
Plataspidae	*Coptosoma parvipictum*	Phyto	2 Rows	—
	Coptosoma sphaerula	Phyto	2 Rows	– (0/3)
	Megacopta punctatissima	Phyto	2 Rows	—
	Megacopta cribraria	Phyto	2 Rows	—
	Brachyplatys subaeneus	Phyto	2 Rows	—
Parastrachiidae	*Parastrachia japonensis*	Phyto	2 Rows	—
Cydnidae	*Adomerus triguttulus*	Phyto	2 Rows	—
	Chilocoris piceus	Phyto	2 Rows	—
	Psamnozetes ater	Phyto	2 Rows	—
	Macroscytus japonensis	Phyto	2 Rows	—
	Adrisa magna	Phyto	2 Rows	—
Scutelleridae	*Cantao ocellatus*	Phyto	4 Rows	—
	Philia miyakonus	Phyto	4 Rows	—
	Calliphara nobilis	Phyto	4 Rows	—
	Calliphara exellens	Phyto	4 Rows	—
	Eucorysses grandis	Phyto	4 Rows	—
	Poecilocoris lewisi	Phyto	4 Rows	—
	Solenosthedium chinense	Phyto	4 Rows	—
Dinidoridae	*Megymenum gracilicorne*	Phyto	4 Rows	—
Pentatomidae	*Gonopsis affinis*	Phyto	4 Rows	—
	Dybowskyia reticulata	Phyto	4 Rows	—
	Scotinophara horvathi	Phyto	4 Rows	—
	Scotinophara lurida	Phyto	4 Rows	—
	Graphosoma rubrolineatum	Phyto	4 Rows	—
	Erthesina fullo	Phyto	4 Rows	– (0/2)
	Aelia fieberi	Phyto	4 Rows	—
	Halyomorpha halys	Phyto	4 Rows	– (0/4)
	Palomena angulosa	Phyto	4 Rows	—
	Dolycoris baccarum	Phyto	4 Rows	– (0/9)
	Eysarcoris lewisi	Phyto	4 Rows	—
	Eysarcoris aeneus	Phyto	4 Rows	—
	Eysarcoris ventralis	Phyto	4 Rows	—
	Eysarcoris annamita	Phyto	4 Rows	– (0/2)
	Carbula humerigera	Phyto	4 Rows	– (0/2)
	Eurydema rugosa	Phyto	4 Rows	—
	Eurydema dominulus	Phyto	4 Rows	—
	Agonoscelis femoralis	Phyto	4 Rows	—
	Plautia stali	Phyto	4 Rows	– (0/6)
	Nezara antennata	Phyto	4 Rows	—
	Homalogonia obtuse	Phyto	4 Rows	—
	Menida violacea	Phyto	4 Rows	—
	Piezodorus hybneri	Phyto	4 Rows	—
	Bathycoelia indica	Phyto	4 Rows	—
	Pentatoma japonica	Phyto	4 Rows	—
	Rhynchocoris humeralis	Phyto	4 Rows	—
	Eocanthecona furcellata	Pred	—	—
	Andrallus spinidens	Pred	—	—
	Zicrona caerulea	Pred	—	—
Acanthosomatidae	*Elasmostethus humeralis*	Phyto	Flat	– (0/7)
	Elasmostethus nubilus	Phyto	Flat	– (0/5)
	Elasmostethus brevis	Phyto	Flat	—
	Elasmucha dorsalis	Phyto	Flat	—
	Elasmucha putoni	Phyto	Flat	—
	Elasmucha amurensis	Phyto	Flat	—
	Elasmucha signoreti	Phyto	Flat	—
	Sastragala esakii	Phyto	Flat	—
	Sastragala scutellata	Phyto	Flat	—
	Acanthosoma denticaudum	Phyto	Flat	—
	Acanthosoma labiduroides	Phyto	Flat	—
	Acanthosoma haemorrhoidale	Phyto	Flat	—

[a]Collection data of the stinkbug samples (collection site, collection date, host plant species, and collector) are shown in Supplementary Table S1.
[b]Myco, mycophagous; Phyto, phytophagous; Pred, predatious.
[c]Tubes, long tubular crypts grown from the midgut fourth section; 2 rows, numerous small crypts fused into two rows along the midgut fourth section; 4 rows, numerous small crypts fused into four rows along the midgut fourth section; Flat, numerous thin and long crypts fused into two large flat assemblages like butterfly wings along the midgut fourth section.
[d]+, *Buekholderia*-positive; −, *Burkholderia*-negative; in parentheses, number of *Burkholderia*-positive insects/number of field-collected insects examined.
[e]Species subjected to DNA extraction and cloning of bacterial 16S rRNA gene. The results are summarized in Table 2.
[f]Species subjected to *Burkholderia* culturing from the midgut crypts. The results are summarized in Table 2.

and a laser scanning confocal microscope (LSCM Pascal5, Carl Zeiss). To confirm specificity of the detection, a series of control experiments were conducted as described (Kikuchi *et al.*, 2005).

Symbiont culture

Field-collected insects were surface-sterilized in 70% ethanol, and individually dissected in phosphate-buffered saline. A midgut region with crypts was carefully cut out, repeatedly washed with fresh sterilized water to minimize possible microbial contamination, and homogenized in 1 ml of fresh sterilized water. An adequate amount of the homogenate was spread on yeast extract and glucose (YG) agar plates (0.5% yeast extract, 0.4% glucose, 0.1% NaCl, 1.5% Bacto agar) and incubated at 26 °C for 3 days or longer. From each of the plates, 15 or more colonies were randomly picked and subjected to PCR, cloning and sequencing of 16S rRNA gene.

Results

General observation of midgut crypts in diverse stinkbugs

We dissected 124 pentatomomorphan stinkbug species and inspected their midgut under a binocular microscope. Development of crypts in the midgut fourth section was observed in 96 species representing 15 families (Table 1). Arrangements of the midgut crypts were distinct among the stinkbug taxa, which were classified into at least four morphological types (Table 1; also see Kikuchi *et al.*, 2008): tubular outgrowths in the Blissidae, Berytidae and most species of the Rhyparochromidae (Figure 2b, c, e, f, h and i); small crypts arranged in two rows in the Coreidae, Alydidae, Plataspidae, Parastrachiidae, Cydnidae, Pachygronthidae and several species of the Rhyparochromidae (Figure 2k, l, n, o, q and r; also see Kikuchi *et al.*, 2005; Hosokawa *et al.*, 2010); small crypts arranged in four rows in the Pentatomidae, Scutelleridae, Urostylidae and Dinidoridae (see Abe *et al.*, 1995; Kaiwa *et al.*, 2010); and a pair of large flat assemblage of crypts in the Acanthosomatidae (see Kikuchi *et al.*, 2009). These results were in agreement with early histological observations of stinkbug alimentary tracts (Glasgow, 1914; Miyamoto, 1961; Buchner, 1965).

Detection of Burkholderia symbionts from diverse stinkbugs

These stinkbug species were subjected to diagnostic PCR using *Burkholderia*-specific primers. Of the 124 species, *Burkholderia* infection was detected from 39 species of the families Berytidae, Blissidae, Rhoparochromidae, Pachygronthidae, Coreidae and Alydidae, which belong to the superfamilies Lygaeoidea and Coreoidea. In these stinkbug groups, notably, *Burkholderia* infection was coincident with development of the midgut crypts: all the 39 species

with the midgut crypts were *Burkholderia*-positive (Table 1; Figure 2), whereas the other 22 species exhibited neither the crypts nor the *Burkholderia* infection (Table 1; Supplememtary Figure S1). The infection frequencies in natural populations were generally high: 91% (10/11) in *Yemma exilis*; 100% (31/31) in *Dimorphopterus pallipes*; 100% (36/36) in *Togo hemipterus*; 100% (18/18) in *Pachygrontha antennata*; 100% (3/3) in *Acanthocoris sordidus*, *Hygia lativentris* and *Homoeocerus unipunctatus* and 88% (22/25) in *Cletus punctiger* (Table 1).

Bacterial 16S rRNA gene sequences from Burkholderia-positive stinkbugs

Of the 39 *Burkholderia*-positive stinkbugs, selected 19 species were subjected to PCR and cloning of bacterial 16S rRNA gene from their midgut crypts. Restriction fragment length polymorphism profiles of the observed 190 clones, 10 clones each from the 19 samples, exhibited identical patterns (data not shown). Three or more clones from each of the samples were subjected to DNA sequencing. All sequences obtained from a single insect were identical, while the sequences from different species slightly (<0.03%) differed from each other. BLAST searches against the DNA databases revealed that the sequences exhibited the highest (>99%) similarities to 16S rRNA gene sequences of *Burkholderia* symbionts from alydid stinkbugs *R. pedestris* and *L. chinensis* (Kikuchi *et al.*, 2005, 2007).

In vivo localization of Burkholderia symbionts

The following representatives of crypt-bearing lygaeoid/coreoid stinkbugs, *D. pallipes* (Blissidae), *T. hemipterus* (Rhyparochromidae), *A. sordidus* (Coreidae) and *L. chinensis* (Alydidae), were dissected and subjected to *in situ* hybridization targeting 16S rRNA of *Burkholderia* symbionts. In all the species, *Burkholderia* signals were consistently localized in the crypts associated with the midgut fourth section (Figure 3). The localization patterns were similar to that described for *R. pedestris* (Alydidae; Kikuchi *et al.*, 2005).

Isolation and culture of Burkholderia symbionts

Considering that the *Burkholderia* symbiont of *R. pedestris* is culturable on standard bacterial media (Kikuchi *et al.*, 2007), we attempted to isolate *Burkholderia* symbionts using YG medium agar plates from the following lygaeoid/coreoid stinkbugs: *Y. exilis* (Berytidae); *D. pallipes* (Blissidae); *P. exiguus* and *T. hemipterus* (Rhyparochromidae); *P. antennata* (Pachygronthidae); *A. sordidus, H. lativentris* and *C. puntiger* (Coreidae); and additionally *R. pedestris* and *L. chinensis* (Alydidae). Within 3 days after plating of homogenized midgut crypts, a number of white, circular and convex colonies appeared for all the stinkbug samples
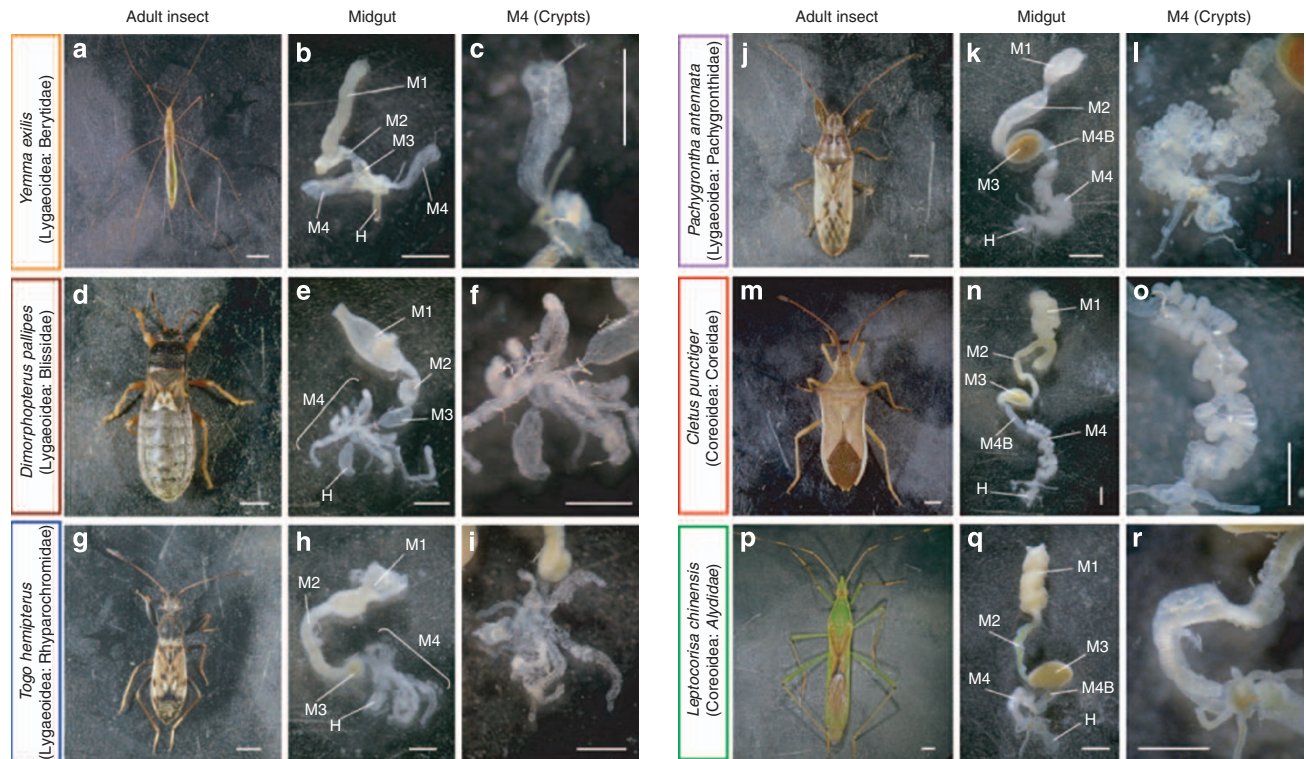
Figure 2 Midgut organization in stinkbugs of the superfamilies Lygaeoidea and Coreoidea. (**a–c**) *Yemma exilis* (Lygaeoidea: Berytidae); (**d–f**) *Dimorphopterus pallipes* (Lygaeoidea: Blissidae); (**g–i**) *Togo hemipterus* (Lygaeoidea: Rhyparochromidae); (**j–l**) *Pachygrontha antennata* (Lygaeoidea: Pachygronthidae); (**m–o**) *Cletus punctiger* (Coreoidea: Coreidae); (**p–r**) *Leptocorisa chinensis* (Coreoidea: Alydidae). Adult female insects, dissected midguts and enlarged images of the midgut crypts are shown from left to right. Bars show 1 mm. Abbreviations: M1, midgut first section; M2, midgut second section; M3, midgut third section; M4, midgut fourth section with crypts; M4B, bulbous section before M4; H, hindgut.

examined (Figure 4a–f). Microscopic observation of bacterial cells from the colonies consistently revealed 1.0–2.0 μm rods (Figure 4g–l). When 15 or more of the white colonies were picked from each plate and subjected to diagnostic PCR, all the colonies were diagnosed as *Burkholderia*-positive. In an individual of *P. antennata*, several yellowish and brownish colonies were exceptionally observed, whose 16S rRNA gene sequences showed high (>99%) sequence similarities to those of *Stenotrophomonas maltophilia* (AM743169) and *Delftia tsuruhatensis* (EF421404), respectively.

Detection of co-infecting Burkholderia *symbionts*
The cultured *Burkholderia* colonies were mostly uniform in morphology. However, it was occasionally found that some colonies obtained from a single insect differed in size and shape. In such cases, two to three colonies were picked for each of the colony types and subjected to PCR and sequencing of 16S rRNA gene. All obtained sequences showed high similarities (97–100%) to 16S rRNA gene sequences of the *Burkholderia* symbionts identified from dissected midgut crypts of the lygaeoid/coreoid stinkbugs as described above. In total, 24 different *Burkholderia* strains were isolated from 16 insects representing 10 species and 6 families, among which 10, 4 and 2 insects were identified as single-, double- and triple-infected, respectively (Table 2).

Molecular phylogenetic analysis of Burkholderia *symbionts*
The 16S rRNA gene sequences of the *Burkholderia* symbionts were subjected to molecular phylogenetic analysis together with *Burkholderia* and allied β-proteobacterial 16S rRNA gene sequences retrieved from the DNA databases. The sequences from the lygaeoid and coreoid stinkbugs formed a group in the genus *Burkholderia*, although the whole assemblage received poor statistical supports (45% for NJ and <40% for ML). In the group, the sequences from the coreid stinkbugs formed a compact and well-supported monophyletic group (100% for both NJ and ML), named the 'Coreidae clade'. The other sequences were placed in a poorly supported clade (75% for NJ and <40% for ML). The clade also contained the sequences of *Burkholderia* symbionts from the alydid stinkbugs *R. pedestris* and *L. chinensis* (Kikuchi *et al.*, 2005, 2007), environmental *Burkholderia* isolates such as strains AK-5, NF100 and others (Hayatsu *et al.*, 2000; Takenaka *et al.*, 2003), and gall-forming *Burkholderia*
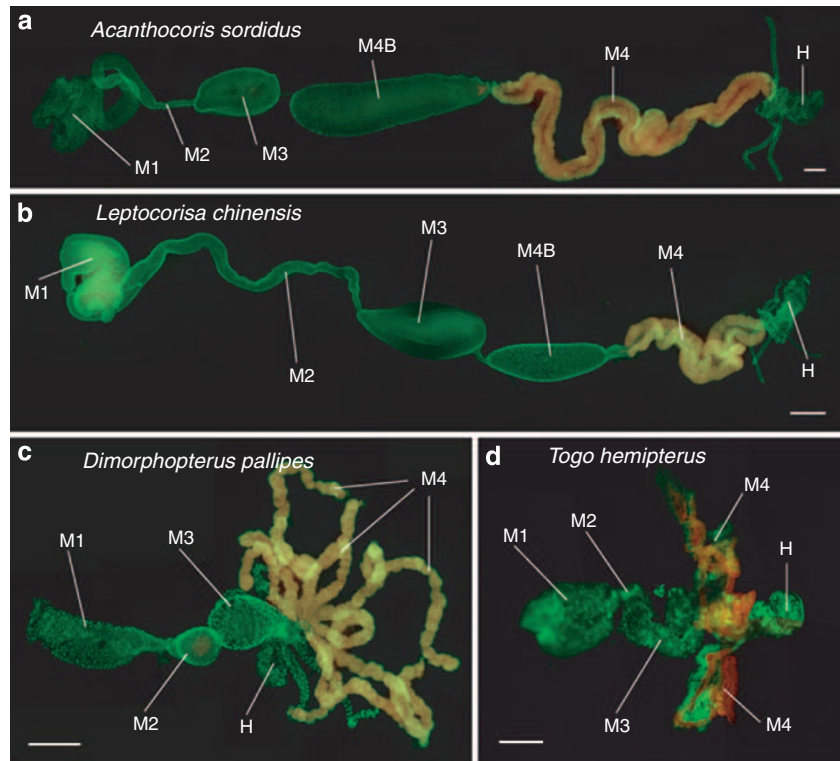
Figure 3 Fluorescent *in situ* hybridization targeting 16S rRNA of *Burkholderia* symbionts in dissected midguts of lygaeoid and coreoid stinkbugs. Red signals indicate *Burkholderia* symbionts, whereas green signals show host insect nuclei. (**a**) *Acanthocoris sordidus* (Coreoidea: Coreidae); (**b**) *Leptocorisa chinensis* (Coreoidea: Alydidae); (**c**) *Dimorphopterus pallipes* (Lygaeoidea: Blissidae); (**d**) *Togo hemipterus* (Lygaeoidea: Rhyparochromidae). Bars show 0.5 mm. Abbreviations: M1, midgut first section; M2, midgut second section; M3, midgut third section; M4, midgut fourth section with crypts; M4B, bulbous section before M4; H, hindgut.

species such as *Candidatus* B. calva, *Candidatus* B. kirkii, *Candidatus* B. nigropunctata and *Candidatus* B. verschuerenii from African *Psychotria* plants (Van Oevelen *et al.*, 2002, 2004). These stinkbug-associated, gall-forming and environmental *Burkholderia* strains were phylogenetically not coherent, but scattered on the phylogeny in an intermingled manner (Figure 5; Supplementary Figure S2).

Detection of Burkholderia *symbionts from eggs*
Eggs of several lygaeoid and coreoid stinkbugs were subjected to diagnostic PCR detection of the *Burkholderia* symbionts. No *Burkholderia* signal was detected from all eggs of *D. pallipes* (Blissidae), *P. exiguus* (Rhyparochromidae), *C. punctiger* and *H. lativentris* (Coreidae), and *R. pedestris* (Alydidae). Only a small fraction of eggs from *T. hemipterus* (Rhyparochromidae) and *L. chinensis* (Alydidae) exhibited a faint signal of *Burkholderia* (Table 3). None of the stinkbug eggs accompanied symbiont capsule-like particles (data not shown).

Discussion

In previous studies, gut symbiotic bacteria of the genus *Burkholderia* have been identified from two alydid stinkbug species, *R. pedestris* and *L. chinensis*

(Kikuchi *et al.*, 2005, 2007). Here, we performed an extensive survey of 124 stinkbug species representing 20 families and 5 superfamilies, and identified *Burkholderia* symbionts from 39 species representing 6 families and 2 superfamilies, namely Lygaeoidea and Coreoidea (Table 1). Irrespective of the stinkbug groups, the *Burkholderia* symbionts were localized in the specialized symbiotic organ, namely a posterior midgut section bearing crypts (Figure 3), and infection frequencies in natural populations were generally high (Table 1). These results indicate that the β-proteobacterial *Burkholderia* symbionts are widely associated with lygaeoid and coreoid stinkbugs.

In the superfamily Pentatomoidea, meanwhile, different groups of stinkbugs are associated with different lineages of γ-proteobacterial midgut symbionts: *Ishikawaella* with the family Plataspidae (Hosokawa *et al.*, 2006), *Rosenkranzia* with the family Acanthosomatidae (Kikuchi *et al.*, 2009), and several γ-proteobacterial lineages with the families Pentatomidae (Prado and Almeida, 2009a), Scutelleridae (Kaiwa *et al.*, 2010) and Parastrachiidae (Hosokawa *et al.*, 2010). In the molecular phylogeny of the pentatomomorphan families, the Lygaeoidea/Coreoidea associated with β-proteobacterial *Burkholderia* symbionts formed a distinct major group from the Pentatomoidea associated with

γ-proteobactertial symbionts (Figure 1; Xie *et al.*, 2005; Hua *et al.*, 2008). These patterns suggest that host–symbiont specificity/compatibility may exist

Figure 4 Colony forms (**a–f**) and bacterial cell images (**g–l**) of *Burkholderia* symbiont strains YEX123 from *Yemma exilis*, DPA58 from *Dimorphopterus pallipes*, THE68 from *Togo hemipterus*, PAN137 from *Pachygrontha antennata*, CPU52 from *Cletus punctiger* and LCH90 from *Leptocorisa chinensis*. Colony formations on YG agar plates were observed after incubation at 26 °C for 3 days. Stationary-phase bacterial cells were observed by phase-contrast microscopy. Bars show 1 mm in (**a–f**) and 10 μm in (**g–l**).

at the higher taxomic levels of the stinkbugs, which has been maintained over evolutionary time.

We demonstrated that in the lygaeoid and coreoid stinkbugs, the universal occurrence of *Burkholderia* does not entail host–symbiont co-speciation. Molecular phylogenetic analyses revealed that the symbiont phylogenies did not reflect the host systematics at all: the same host species frequently harbored different symbiont genotypes, and different host species often possessed nearly or completely identical symbiont genotypes (Figure 5; Supplementary Figure S2). These patterns strongly suggest that host–symbiont associations are generally promiscuous in the Lygaeoidea and the Coreoidea. The situation looks different from the situation in the Pentatomoidea, wherein host–symbiont associations are, if not strict, more or less stable. In the families Plataspidae and Acanthosomatidae, respectively, the symbiont phylogenies are highly congruent with the host phylogenies, indicating stable vertical transmission and co-speciation of their gut symbionts over evolutionary time (Hosokawa *et al.*, 2006; Kikuchi *et al.*, 2009). In the family Pentatomidae, although their gut symbionts are presumably of multiple evolutionary origins, the symbionts are generally conserved within the same species and sometimes between congenic species (Prado and Almeida, 2009a).

Previous works showed that, in different groups of the Pentatomoidea, γ-proteobactertial gut symbionts are vertically transmitted through host generations (Schorr, 1957; Abe *et al.*, 1995; Hosokawa *et al.*, 2005; Prado *et al.*, 2006; Kikuchi *et al.*, 2009; Prado and Almeida, 2009b; Kaiwa *et al.*, 2010). In this study, inspection of eggs of lygaeoid and coreoid stinkbugs revealed no stable vertical transmission of the *Burkholderia* symbionts to their eggs (Table 3). The host–symbiont phylogenetic promiscuity (Figure 5; Supplementary Figure S2) taken together, we suggest that in the lygaeoid and coreoid stinkbugs, their *Burkholderia* gut symbionts are generally acquired from the environment every generation. Such host–symbiont phylogenetic promiscuity is commonly found in the symbiotic associations without vertical transmission, such as coral-dinoflagellate, legume-*Rhizobium*, and legume-*Burkholderia* symbioses (Young and Haukka, 1996; Baker, 2003; Bontemps *et al.*, 2010). It should be noted that occasional vertical transmission of the
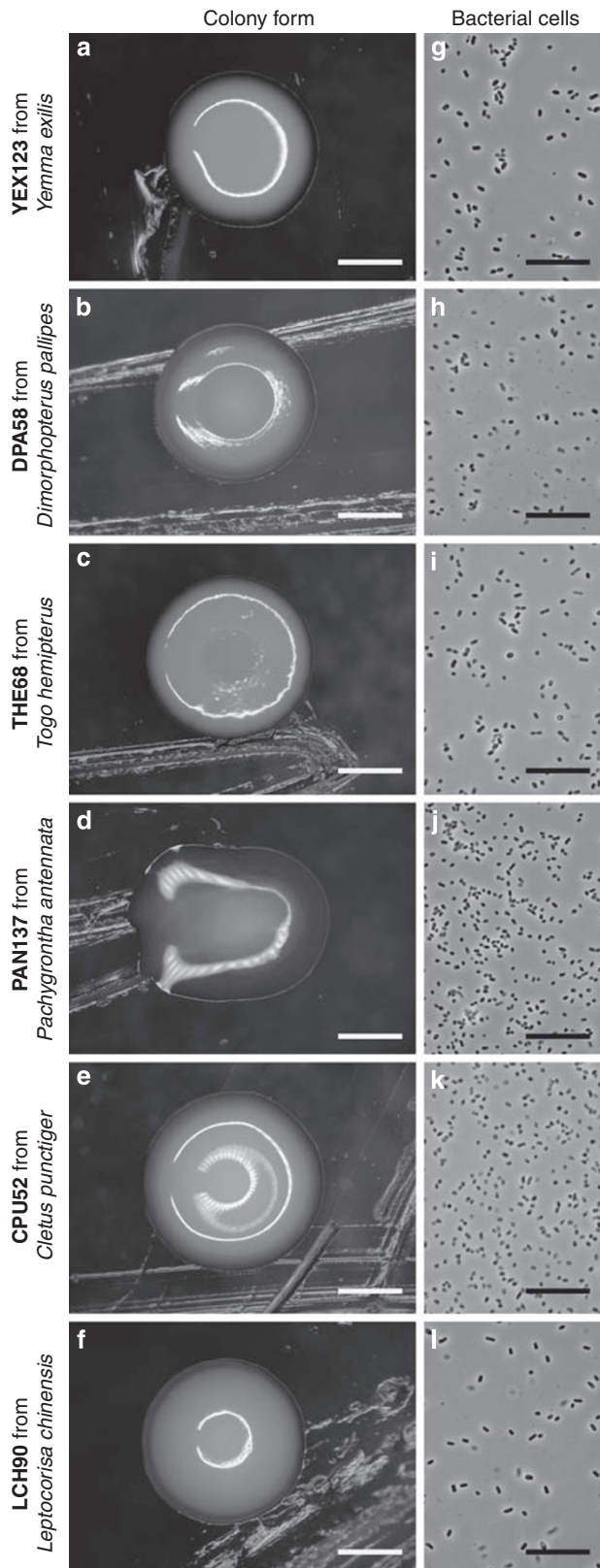
Table 2 *Burkholderia* symbiont strains identified in this study

Superfamily Family	Species	Individual ID[a]	Detection method[b]	Cultured strain ID[c]	Accession number[d]
Lygaeoidea					
Berytidae	*Yemma exilis*	YEX#1	Cloning	—	AB558174
		YEX#2[e]	Plating	YEX123	AB558175
				YEX124	AB558176
Blissidae	*Dimorphopterus pallipes*	DPA#1	Cloning	—	AB558177
		DPA#2	Plating	DPA58	AB558178
		DPA#3	Plating	DPA61	AB558179
Rhyparo-chromidae	*Paromius exiguous*	PEX#1	Cloning	—	AB558180
		PEX#2[e]	Plating	PEX84	AB558181
				PEX86	AB558182
	Togo hemipterus	THE#1	Cloning	—	AB558183
		THE#2	Plating	THE68	AB558184
	Panaorus japonicus	PJA#1	Cloning	—	AB558185
Pachygron-thidae	*Pachygrontha antennata*	PAN#1	Cloning	—	AB558186
		PAN#2	Plating	PAN133	AB558187
		PAN#3[f]	Plating	PAN135	AB558188
				PAN136	AB558189
				PAN137	AB558190
Coreoidea					
Coreidae	*Molipteryx fuliginosa*	MFU#1	Cloning	—	AB558191
	Acanthocoris sordidus	ASO#1	Cloning	—	AB558192
		ASO#2	Plating	ASO24	AB558193
		ASO#3	Plating	ASO50	AB558194
	Notobitus Meleagris	NME#1	Cloning	—	AB558195
	Hygia opaca	HOP#1	Cloning	—	AB558196
	Hygia lativentris	HLA#1	Cloning	—	AB558197
		HLA#2[f]	Plating	HLA54	AB558198
				HLA55	AB558199
				HLA56	AB558200
	Homoeoceus dilatatus	HDI#1	Cloning	—	AB558201
	Homoeocerus unipunctatus	HUN#1	Cloning	—	AB558202
	Plinachtus bicoloripes	PBI#1	Cloning	—	AB558203
	Cletus trigonus	CTR#1	Cloning	—	AB558204
	Cletus punctiger	CPU#1	Cloning	—	AB558205
		CPU#2	Plating	CPU52	AB558206
	Cletus rusticus	CRU#1	Cloning	—	AB558207
Alydidae	*Riptortus pedestris*	RPE#1[e]	Plating	RPE64	AB558208
				RPE65	AB558209
		RPE#2[e]	Plating	RPE66	AB558210
				RPE67	AB558211
	Riptortus linearis	RLI#1	Cloning	—	AB558212
	Leptocorisa chinensis	LCH#1	Plating	LCH76	AB558213
		LCH#2	Plating	LCH78	AB558214
		LCH#3	Plating	LCH90	AB558215
	Daclera levana	DLE#1	Cloning	—	AB558216

[a]Indicating insect individuals examined for their *Burkholderia* symbionts.
[b]Cloning: midgut fourth section dissected from an acetone-preserved insect was subjected to DNA extraction, PCR amplification and cloning of bacterial 16S rRNA gene. Plating: midgut fourth section dissected from a fresh insect was homogenized and plated on YG medium agar plates, and bacterial colonies on the plates were subjected to DNA extraction, PCR amplification and cloning of bacterial 16S rRNA gene.
[c]The cultured bacterial strains are maintained by the corresponding author (Y Kikuchi) and available on request.
[d]The nucleotide sequences of 16S rRNA gene determined in this study were deposited in the DNA Data Bank of Japan (DDBJ) with the accession numbers.
[e]Double infection with *Burkholderia* symbionts.
[f]Triple infection with *Burkholderia* symbionts.

symbiont via, for example, accidental fecal contamination of eggs (see Table 3; Huber-Schneider, 1957) is compatible with the environmental acquisition of the symbiont in the same species. Needless to say, the presumable prevalence of the environmental symbiont acquisition should be experimentally verified in each of the lygaeoid and coreoid species, as shown in *R. pedestris* (Kikuchi *et al.*, 2007).

The global stability and the local promiscuity of the host–symbiont associations in the pentatomomorphan stinkbugs are of evolutionary interest. The host–symbiont promiscuity in the lygaeoid and coreoid stinkbugs must be due to the environmental symbiont acquisition. The polyphyly of the vertically-transmitted symbionts in the pentatomoid stinkbugs are probably caused by occasional horizontal transfers of the symbionts across distinct host
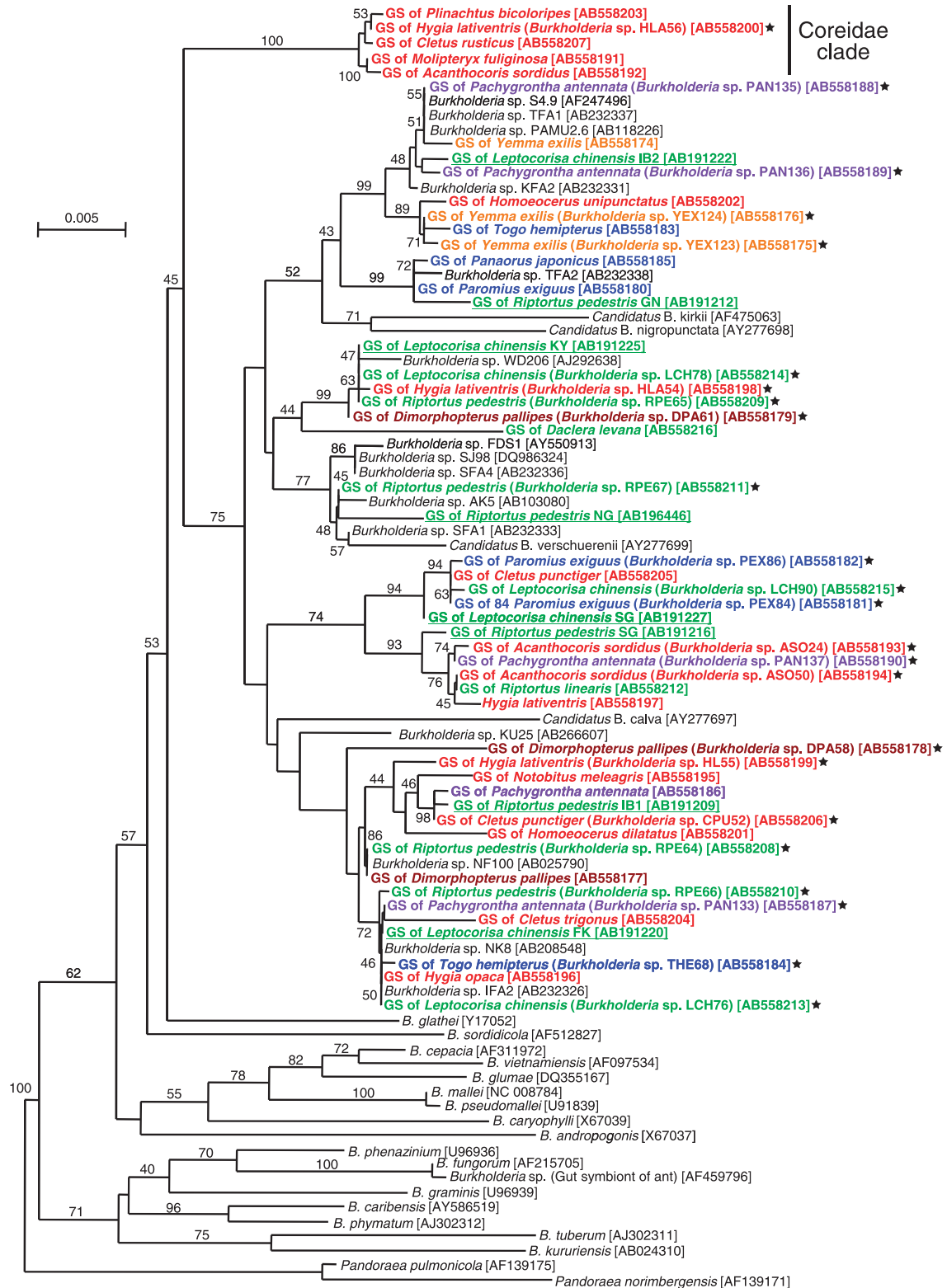
Figure 5 Phylogenetic relationship of 43 *Burkholderia* symbiont genotypes identified from 21 stinkbug species of the superfamilies Lygaeoidea and Coreoidea on the basis of 16S rRNA gene sequences. A neighbor-joining tree, inferred from 1228 unambiguously aligned nucleotide sites, is shown. The *Burkholderia* symbionts were detected from the stinkbug families Berytidae (orange), Blissidae (brown) Rhyparochromidae (blue) and Pachygronthidae (purple) of the superfamily Lygaeoidea, and the stinkbug families Coreidae (red) and Alydidae (green) of the superfamily Coreoidea. Stars indicate cultured strains of the *Burkholderia* symbionts, whose strain identifiers are shown in parentheses (also see Table 2). The sequences from *Riptortus pedestris* and *Leptocorisa chinensis* reported in a previous study (Kikuchi *et al.*, 2005) are underlined. The bootstrap values >40% are depicted at the nodes. In brackets are shown nucleotide sequence accession numbers. Abbreviation: GS, gut symbiont.

Table 3 Detection of *Burkholderia* symbionts from eggs

Superfamily family	Species	Burkholderia detection[a]
Lygaeoidea		
Blissidae	*Dimorphopterus pallipes*	0% (0/26)
Rhyparochromidae	*Paromius exiguus*	0% (0/28)
	Togo hemipterus	4.9% (2/41)[b]
Coreoidea		
Coreidae	*Hygia lativentris*	0% (0/12)
	Cletus punctiger	0% (0/24)
Alydidae	*Riptortus pedestris*	0% (0/144)[c]
	Leptocorisa chinensis	6.6% (3/45)[b]

[a]Detection percentage (number of *Burkholderia*-positive eggs/total number of eggs examined).
[b]Exhibiting a faint band after 35 cycles of PCR.
[c]Data from Kikuchi *et al.* (2007).

lineages. Here it is puzzling why the lygaeoid and coreoid stinkbugs remain associated with the β-proteobacterial symbionts despite the intensive host–symbiont mixing at lower levels, so do the pentatomoid stinkbugs with the γ-proteobacterial symbionts. Although speculative, the host–symbiont affinities mediated by their surface molecules might be relevant to the mechanisms for specific recognition and establishment of the symbiosis at the midgut crypts.

Biological roles of the *Burkholderia* symbionts for the lygaeoid and coreoid stinkbugs have been poorly understood. The only experimental proof was obtained for the alydid species *R. pedestris*, wherein aposymbiosis resulted in reduced body size (Kikuchi *et al.*, 2007). On account of the specific localization in highly developed symbiotic organ (Figure 2) and the universal occurrence and conservation among the diverse lygaeoid and coreoid species (Table 1), it appears likely that the *Burkholderia* symbionts generally have important roles for their insect hosts. Considering that all the *Burkholderia*-associated stinkbugs were phytophagous (Table 1), their symbionts might provide nutritional components such as vitamins and essential amino acids that are often deficient in the plant sap diet. Some *Burkholderia* strains exhibit nitrogen-fixing ability (Estrada-De *et al.*, 2001), and the *Burkholderia* symbionts might contribute nitrogenous metabolites to their stinkbug hosts. On account of the taxonomic breadth and diversity of the lygaeoid and coreoid stinkbugs, the effects of the *Burkholderia* symbionts might be different from species to species, possibly ranging from commensalistic through beneficial to essential for their insect hosts. In this context, the *Burkholderia* symbionts of the 'Coreidae clade' (Figure 5; Supplementary Figure S2) are of particular interest, because the distinct symbiont lineage is specifically associated with coreid species, representing an exception of the generally promiscuous

host–symbiont associations in the lygaeoid and coreoid stinkbugs. We suggest the possibility that an advanced form of stinkbug-*Burkholderia* mutualism would be identified in these coreid species.

In this study, we found that not all the lygaeoid and coreoid species harbor the *Burkholderia* gut symbionts. In the Lygaeoidea, stinkbugs of the Berytidae, Blissidae, Rhyparochromidae and Pachygronthidae were associated with the *Burkholderia* symbionts, whereas species of the Malcidae, Lygaeidae and Geocoridae were not. In the Coreoidea, stinkbugs of the Coreidae and Alydidae possessed the *Burkholderia* symbionts, whereas species of the Rhopalidae did not (Table 1). It is currently elusive what ecological, physiological and/or phylogenetic factors are relevant to the presence/absence of the *Burkholderia* symbionts. It should be noted that, in all the lygaeoid and coreoid stinkbugs, the presence of the *Burkholderia* symbiont was coincident with the presence of crypts in the posterior midgut (Figure 2; Supplementary Figure S1; Table 1), suggesting that the specialized morphological configuration is pivotal for establishment and maintenance of the symbiotic association. As the presence/absence of the crypts/*Burkholderia* reflected the host insect systematics (Table 1), it appears plausible that the symbiotic trait is mainly determined by the host genotype. On the other hand, the possibility cannot be ruled out that the *Burkholderia* infection is also involved in the formation of the symbiotic structure in the host midgut. In leguminous plants, infection with symbiotic *Rhizobium* induces nodule formation in the root (Perret *et al.*, 2000; Oldroyd and Downie, 2008). In *Euprymna* squids, infection with luminescent *Vibrio fischeri* is required for normal morphogenesis of the symbiotic light organ (McFall-Ngai and Ruby, 1991; Nyholm and McFall-Ngai, 2004). Whether or not the *Burkholderia* symbiont is involved in the formation of the host symbiotic organ is of interest, deserving future experimental and developmental studies on symbiotic and aposymbiotic insects.

The soil environment harbors an enormous diversity of microorganisms (Garbeva *et al.*, 2004), wherein the fraction of *Burkholderia* symbionts must be very small. Hence, it is expected that the nymphs of lygaeoid and coreoid stinkbugs orally acquire diverse microorganisms, from which the *Burkholderia* symbiont is selected to establish specific infection in the midgut crypts. In symbiotic associations entailing environmental transmission in general, the host must have some mechanisms for enrichment and harvesting of specific microorganisms. Once the symbiosis is established, there must be mechanisms to ensure that the association is stable so that the symbionts do not overgrow the host and the host does not eliminate the symbiont (Nyholm and McFall-Ngai, 2004). In the squid–*Vibrio* and legume–*Rhizobium* symbioses, intricate cellular and molecular crosstalks have been shown to underlie the establishment and maintenance of

the symbiotic associations (Perret *et al.*, 2000; Koropatnick *et al.*, 2004; Nyholm and McFall-Ngai, 2004; Oldroyd and Downie, 2008). In the stinkbug–*Burkholderia* symbiosis, biochemical and transcriptomic analyses of the midgut crypts would provide insights into what molecular and cellular processes are operating at the host–symbiont interface.

PCR, cloning and sequencing of 16S rRNA gene from a dissected midgut identified a single sequence type, indicating that a single symbiont genotype tends to dominate the gut microbiota in each of the *Burkholderia*-infected lygaeoid and coreoid stinkbugs. However, culturability of the *Burkholderia* symbiont enabled identification of hidden diversity of the symbiont genotypes coexisting in the same insects: infrequent colonies of different morphotypes on agar plates often represented different *Burkholderia* genotypes (Table 2). Such multiple infections seem to be a natural outcome of the environmental symbiont acquisition, which are also found in other systems such as legume–*Rhizobium*, coral–dinoflagellate and tubeworm–chemoautotroph mutualisms (Moawad and Schmidt, 1987; Rowan *et al.*, 1997; Baker, 2003; Dubilier *et al.*, 2008).

The consequences of hosting multiple symbionts have been a recurrent theme in the evolutionary biology of mutualism. Such multiple infections create a potential tragedy of the commons (Hardin, 1968), which can destabilize and collapse mutualism because within-host competitions tend to favor less beneficial, cheater genotypes (Bull, 1994; West *et al.*, 2002). Hence, it is of interest why mutualisms can be maintained under the absence of vertical transmission and the frequent mixing of different symbiont genotypes. A possible mechanism is so-called 'partner choice', wherein the host recognizes benevolent symbiont genotypes and selectively establishes association with them. Another possible mechanism is so-called 'sanctions', wherein the host monitors the performance of different symbiont genotypes and punishes less beneficial ones (Maynard Smith and Szathmáry, 1995; Wilkinson and Sherratt, 2001; Kiers and Denison, 2008). In the soybean–*Rhizobium* association, an experimental support for host sanctions against less beneficial symbionts has been provided (Kiers *et al.*, 2003). On the soybean root, a number of nodules are formed, each of which may represent a symbiont genotype. Such structured distribution of different symbiont genotypes enables effective operation of host sanctions. In this context, we point out that numerous crypts in the posterior midgut of stinkbugs are regarded as similar structural compartments. It is of interest whether different *Burkholderia* genotypes are separately harbored in different crypts, and whether the crypts harboring different symbiont genotypes tend to exhibit different levels of growth and development.

Overall, the findings presented in this study illuminate dazzlingly promiscuous host–symbiont associations between the diverse lygaeoid/coreoid stinkbugs and the diverse *Burkholderia* genotypes. It is hypothesized that multiple host species may interact with multiple symbiont genotypes, potentially exchanging the partners every generation as follows: different *Burkholderia* genotypes are present in the soil environment, are acquired by nymphal lygaeoid/coreoid stinkbugs, proliferate in the midgut crypts, are released from the insects via excrement or cadaver, spend a free-living life in the soil, and are acquired by nymphal stinkbugs of the same or different species, and so forth. Of course, there must be some permissive range in the host–symbiont associations. Each of the stinkbug species may either be more prone to infection, or perform better, with specific *Burkholderia* genotypes, and *vice versa*. In the enormous space of possible host–symbiont combinations, the host–symbiont permissive ranges may be stratified, overlapped and nested in a complicated manner. As the environmental symbiont acquisition requires host–symbiont encounters, the symbiotic associations must be affected by the habitat and the ecology of the respective symbiotic partners, which are influenced by environmental factors such as geography, climate and vegetation. Here, despite the many-to-many rather than one-to-one relationships, the lygaeoid/coreoid stinkbugs and the *Burkholderia* bacteria have established a form of mutualism, which has been maintained, if not so strictly, during the evolutionary course of the insect superfamilies. Such ecological and evolutionary perspectives are strikingly different from those conventionally inferred from the well-studied insect–microbe endosymbioses represented by aphid–*Buchnera* and tsetse–*Wigglesworthia* relationships, which broadens our understanding of possible evolutionary trajectories leading to stable mutualistic associations.

Acknowledgements

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