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ORIGINAL ARTICLE

# Evolution of symbiotic organs and endosymbionts in lygaeid stinkbugs

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**We investigated seed bugs of the genus *Nysius* (Insecta: Hemiptera: Lygaeidae) for their symbiotic bacteria. From all the samples representing 4 species, 18 populations and 281 individuals, specific bacterial 16S rRNA gene sequences were consistently identified, which formed a distinct clade in the *Gammaproteobacteria*. *In situ* hybridization showed that the bacterium was endocellularly localized in a pair of large bacteriomes that were amorphous in shape, deep red in color, and in association with gonads. In the ovary of adult females, the endosymbiont was also localized in the 'infection zone' in the middle of each germarium and in the 'symbiont ball' at the anterior pole of each oocyte, indicating vertical transmission of the endosymbiont through the ovarial passage. Phylogenetic analyses based on bacterial 16S rRNA, *groEL* and *gyrB* genes consistently supported a coherent monophyly of the *Nysius* endosymbionts. The possibility of a sister relationship to '*Candidatus Kleidoceria schneideri*', the bacteriome-associated endosymbiont of a lygaeid bug *Kleidocerys resedae*, was statistically rejected, indicating independent evolutionary origins of the endosymbionts in the Lygaeidae. The endosymbiont genes consistently exhibited AT-biased nucleotide compositions and accelerated rates of molecular evolution, and the endosymbiont genome was only 0.6 Mb in size. The endosymbiont phylogeny was congruent with the host insect phylogeny, suggesting strict vertical transmission and host–symbiont co-speciation over evolutionary time. Based on these results, we discuss the evolution of bacteriomes and endosymbionts in the Heteroptera, most members of which are associated with gut symbiotic bacteria. The designation '*Candidatus Schneideria nysicola*' is proposed for the endosymbiont clade.**
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**Keywords:** stinkbug; *Nysius*; Lygaeidae; bacteriome; endosymbiont

## Introduction

Symbiotic bacteria are universally associated with a diverse array of insects (Buchner, 1965; Bourtzis and Miller, 2003). Among them, the most intimate mutualistic associations are found in obligate endosymbionts like *Buchnera* in aphids and *Wigglesworthia* in tsetse flies, wherein the symbiotic bacteria are harbored in specialized cells called bacteriocytes. In the body of these insects, the bacteriocytes often form a symbiotic organ called bacteriome, where the endosymbionts play their biological roles such as provisioning of essential nutrients (Douglas, 1998; Baumann, 2005). Early histological studies described the formation processes of the bacteriocytes and the bacteriome in aphids, tsetse flies, lice and many other insects (reviewed in Buchner, 1965). However, recent studies using modern molecular and cytological techniques are scarce, except for those on the development of bacteriocytes in the pea aphid *Acyrthosiphon pisum* (Braendle *et al.*, 2003; Miura *et al.*, 2003). The evolutionary origin of the novel organ for symbiosis is unknown.

The suborder Heteroptera is a group of insects with a sucking mouthpart, the so-called true bugs or stinkbugs. The infraorder Pentatomomorpha is placed under the Heteroptera, and contains over 12 500 species that are grouped into five superfamilies (Schuh and Slater, 1995; Henry, 1997). Besides relatively minor predacious and mycophagous species, the majority of them are plant sap feeders, and most of them possess a number of sac- or tube-like outgrowths, called crypts or caeca, in a posterior region of the midgut, whose cavity harbors specific bacterial symbionts (Buchner, 1965; Kikuchi *et al.*, 2008). In the superfamily

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Pentatomoidea, their gut symbionts belong to distinct lineages of the *Gammaproteobacteria*, and are vertically transmitted through host generations (Fukatsu and Hosokawa, 2002; Prado *et al.*, 2006; Hosokawa *et al.*, 2006, 2010; Kikuchi *et al.*, 2009; Prado and Almeida, 2009a, b; Kaiwa *et al.*, 2010, 2011). On the other hand, in the superfamilies Lygaeoidea and Coreoidea, their gut symbionts belong to the β-proteobacterial genus *Burkholderia*, and are acquired from the environmental bacterial pool every host generation (Kikuchi *et al.*, 2005, 2007, 2011). In summary, extracellular symbiotic associations with specific bacteria in midgut crypts are regarded as the default symbiotic system generally found among diverse stinkbugs.

However, Schneider (1940) described a few lygaeoid stinkbugs that exceptionally host endosymbiotic bacteria within specialized bacteriomes. *Nysius punctipennis*, *N. senecionis*, *N. thymi* and *N. lineolatus* (Lygaeidae: Orsillinae) possessed paired red bacteriomes associated with the gonads, *Kleidocerys* (=*Ischnorrhynchus*) *resedae* and *K. ericae* (Lygaeidae: Ischnorrhynchinae) were with an unpaired bacteriome associated with the midgut and *Ischnodemus sabuleti* (Blissidae) was with a pair of bacteriomes in the fat body. Considering that most of the other lygaeoid groups are consistently associated with extracellular *Burkholderia* symbionts in the midgut crypts (Kikuchi *et al.*, 2011), the exceptional lygaeoid stinkbugs harboring the bacteriome-associated endosymbiont are of great interest. These lygaeoid stinkbugs would provide novel insights into the evolutionary origin of the symbiotic organ.

Since the pioneer work by Schneider (1940), neither the bacteriomes nor the endosymbionts of the lygaeoid stinkbugs have been investigated. Recently, the endosymbiont of *K. resedae* was microbiologically characterized and designated as '*Candidatus Kleidoceria schneideri*' (Küchler *et al.*, 2010). Here we report a series of comprehensive analyses on the bacteriome-associated endosymbionts of another lygaeoid group, seed bugs of the genus *Nysius* representing 4 species, 18 populations and 281 individuals, using multidisciplinary approaches from morphology, histology, PCR diagnosis, molecular phylogenetic and evolutionary analyses, *in situ* hybridization, electron microscopy and pulsed-field gel electrophoresis.

## Materials and methods

*Insect materials*

Table 1 lists insect samples of *Nysius* and other lygaeid species used in this study. Most of the samples were preserved in acetone upon collection for DNA analyses (Fukatsu, 1999). Some of the insects were dissected and subjected to *in situ* hybridization, electron microscopy or pulsed-field gel electrophoresis.

*DNA extraction, PCR, cloning, genotyping and sequencing procedures*

Adult insects were dissected in a phosphate-buffered saline with fine forceps under a dissection microscope, and their bacteriomes, ovaries and midguts were isolated. Each of the insect tissues was homogenized and digested in a 1.5 ml plastic tube with a proteinase K-containing lysis buffer, extracted with phenol–chloroform and subjected to ethanol precipitation of whole nucleic acids. Bacterial 16S rRNA, *groEL* and *gyrB* genes were amplified by PCR from the DNA samples, and subjected to cloning, restriction fragment length polymorphism genotyping and sequencing as described (Kikuchi *et al*, 2009). Insect mitochondrial *cytochrome oxidase I* (*COI*) gene, which has been widely used for phylogenetic characterization of insects and other organisms (Hebert *et al.*, 2003), was also amplified by PCR and subjected to direct sequencing. The primers and PCR conditions are listed in Supplementary Table S1. The primers and PCR conditions used for diagnostic PCR are also listed in Supplementary Table S1.

*Molecular phylogenetic, molecular evolutionary and genomic analyses*

Multiple alignments of nucleotide sequences were generated with the program Vector NTI Advance 10.3.1 (Invitrogen, Carlsbad, CA, USA). Phylogenetic analyses were conducted by maximum likelihood, maximum parsimony and neighbor-joining methods. Maximum likelihood trees were generated with the program PhyML 3.0 (Guindon and Gascuel, 2003), whereas maximum parsimony and neighbor-joining trees were constructed with the program MEGA 4 (Tamura *et al.*, 2007). In the maximum likelihood analyses, models were chosen on the basis of the Akaike information criterion with the programs Modeltest 3.7 (Posada and Crandall, 1998) and PAUP* Version 4.0b10 (Swofford, 2001), which selected the GTR + I + G model for each of the 16S rRNA, *groEL*, *gyrB* and *COI* gene phylogenies. Bootstrap tests were conducted with 100 replications. Relative rate tests were performed with the program RRTree (Robinson-Rechavi and Huchon, 2000). The phylogenetic affinity between the endosymbionts of *Nysius* spp. and the endosymbiont of *K. resedae* was evaluated by an approximately unbiased test (Shimodaira, 2002) using the concatenated alignment of 16S rRNA, *groEL* and *gyrB* gene sequences as a single data set. The nucleotide sequences from 11 endosymbionts and 2 free-living bacteria were organized into 9 topological units, the log-likelihood scores for 131 135 possible trees for the topological units were calculated and the best 1000 trees were subjected to the analysis. Log-likelihood scores for all trees and site-wise log-likelihoods for the selected 1000 trees were estimated using RAxML (Stamatakis, 2006). The data were used as input for CONSEL 0.1 g (Shimodaira and Hasegawa, 2001) to calculate

**Table 1** Samples of lygaeid stinkbugs, detection of their symbionts, and accession numbers of nucleotide sequences determined in this study.

| *Taxon* | *Locality*[a] | *Date*[b] | *Collector* | *Host plant* | *Symbiotic organ* | | *Symbiont detection*[c] | | *Sequence accession number* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Schneideria[d] | Burkholderia | *16S rRNA* | gyrB | groEL | COI |
| Family Lygaeidae | | | | | | | | | | | | |
| Subfamily Orsillinae | | | | | | | | | | | | |
| *Nysius plebeius* | Tsukuba, Ibaraki | 11/09 /09 | YM | *Conyza sumatrensis* | Paired bacteriome | ♀ | 100% (37/37) | 0% (0/37) | AB576918 | AB583935 | AB583951 | AB619226 |
| | | | | | | ♂ | 100% (26/26) | 0% (0/26) | | | | |
| | Mito, Ibaraki | 10/09 /09 | YK | *C. sumatrensis* | | ♀ | 100% (14/14) | 0% (0/14) | AB576919 | AB583937 | AB583952 | AB619227 |
| | | | | | | ♂ | 100% (7/7) | 0% (0/7) | | | | |
| | Fukuyama, Hiroshima | 22/09 /09 | YM | *C. sumatrensis* | | ♀ | 100% (5/5) | 0% (0/5) | AB576920 | AB583938 | — | AB619228 |
| | | | | | | ♂ | 100% (7/7) | 0% (0/7) | | | | |
| | Takamatsu, Kagawa | 16/09 /09 | YK | *C. sumatrensis* | | ♀ | 100% (2/2) | 0% (0/2) | AB576921 | AB583939 | AB583953 | AB619229 |
| | | | | | | ♂ | | | | | | |
| | Koshi, Kumamoto | 28/09 /09 | YK | *C. sumatrensis* | | ♀ | 100% (3/3) | 0% (0/3) | AB576922 | AB583940 | AB583954 | AB619230 |
| | | | | | | ♂ | 100% (3/3) | 0% (0/3) | | | | |
| | Tokunoshima, Kagoshima | 30/09 /09 | TH | *C. sumatrensis* | | ♀ | 100% (4/4) | 0% (0/4) | AB576923 | AB583941 | — | AB619231 |
| | | | | | | ♂ | 100% (6/6) | 0% (0/6) | | | | |
| | Ishigakijima, Okinawa | 09/09 /09 | TH | *Chamaesyce hirta* | | ♀ | 100% (15/15) | 0% (0/15) | AB576924 | AB583942 | AB583955 | AB619232 |
| | | | | | | ♂ | 100% (15/15) | 0% (0/15) | | | | |
| | Iriomotejima, Okinawa | 10/09 /09 | TH | *C. hirta* | | ♀ | 100% (3/3) | 0% (0/3) | AB576925 | AB583943 | AB583956 | AB619233 |
| | | | | | | ♂ | 100% (3/3) | 0% (0/3) | | | | |
| | Kumejima, Okinawa | 10/04 /10 | TH | *Youngia* sp. | | ♀ | 100% (1/1) | 0% (0/1) | AB576926 | — | — | AB619234 |
| | | | | | | ♂ | | | | | | |
| *Nysius* sp. 1 | Tsukuba, Ibaraki | 12/09 /09 | YM | *C. sumatrensis* | Paired bacteriome | ♀ | 100% (23/23) | 0% (0/23) | AB576927 | AB583944 | AB583957 | AB619235 |
| | | | | | | ♂ | 100% (20/20) | 0% (0/20) | | | | |
| | Mito, Ibaraki | 10/09 /09 | YK | *C. sumatrensis* | | ♀ | 100% (11/11) | 0% (0/11) | AB576928 | AB583945 | — | AB619236 |
| | | | | | | ♂ | 100% (4/4) | 0% (0/4) | | | | |
| | Takamatsu, Kagawa | 16/09 /09 | YK | *C. sumatrensis* | | ♀ | 100% (4/4) | 0% (0/4) | AB576929 | AB583946 | AB583958 | AB619237 |
| | | | | | | ♂ | 100% (7/7) | 0% (0/7) | | | | |
| | Shimanto, Kochi | 16/09 /09 | YK | *C. sumatrensis* | | ♀ | 100% (3/3) | 0% (0/3) | AB576930 | AB583947 | — | AB619238 |
| | | | | | | ♂ | 100% (4/4) | 0% (0/4) | | | | |
| | Koshi, Kumamoto | 28/09 /09 | YK | *C. sumatrensis* | | ♀ | 100% (2/2) | 0% (0/2) | AB576931 | AB583948 | — | AB619239 |
| | | | | | | ♂ | 100% (2/2) | 0% (0/2) | | | | |
| *Nysius* sp. 2 | Yakushima, Kagoshima | 14/05 /10 | TH | *Erigeron philadelphicus* | Paired bacteriome | ♀ | 100% (2/2) | 0% (0/2) | — | — | — | AB619240 |
| | | | | | | ♂ | 100% (8/8) | 0% (0/8) | | | | |
| | Kunigami, Okinawa | 09/06 /10 | TH | *E. philadelphicus* | | ♀ | 100% (4/4) | 0% (0/4) | — | — | — | AB619241 |
| | | | | | | ♂ | 100% (1/1) | 0% (0/1) | | | | |
| | Kumejima, Okinawa | 11/04 /10 | TH | *Youngia* sp. | | ♀ | 100% (2/2) | 0% (0/2) | AB576932 | AB583949 | AB583959 | AB619242 |
| | | | | | | ♂ | | | | | | |
| *Nysius expressus* | Sapporo, Hokkaido | 02/09 /09 | YM | *E. philadelphicus* | Paired bacteriome | ♀ | 100% (16/16) | 0% (0/16) | AB576933 | AB583950 | AB583960 | AB619243 |
| | | | | | | ♂ | 100% (17/17) | 0% (0/17) | | | | |
| Subfamily Lygaeinae | | | | | | | | | | | | |
| *Spilostethus hospes* | Ishigakijima, Okinawa | 09/09 /09 | TH | *Chamaesyce hirta* | Absent | | Negative | Negative | | | | AB619244 |
| *Oncopeltus nigriceps* | Ishigakijima, Okinawa | 05/07 /02 | KK | *Calotropis gigantean* | Absent | | Negative | Negative | | | | AB619246 |
| *Graptostethus servus* | Ishigakijima, Okinawa | 02/10 /10 | TH | *Carissa macrocarpa* | Absent | | Negative | Negative | | | | — |
| *Tropidothorax belongolowi* | Koshi, Kumamoto | 09/27 /09 | YK | *C. sumatrensis* | Absent | | Negative | Negative | | | | — |
| *Arocatus sericans* | Takashima, Shiga | 28/08 /10 | SK | (sweeping) | Absent | | Negative | Negative | | | | AB619254 |

**Table 1** (Continued)

| Taxon | Locality[a] | Date[b] | Collector | Host plant | Symbiotic organ | Symbiont detection[c] | | Sequence accession number | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Schneideria[d] | Burkholderia | *16S rRNA* | gyrB | groEL | COI |
| *Arocatus melanostomus* | Koka, Shiga | 29/08 /10 | SK | *Clematis apiifolia* | Absent | Negative | Negative | | | | AB619255 |
| Subfamily Ischnorhynchinae | | | | | | | | | | | |
| *Kleidocerys resedae* | Sapporo, Hokkaido | 09/08 /10 | YK | *Betula platyphylla* | A single bacteriome | Negative | Negative | AB585972 | AB619224 | AB583961 | AB619247 |
| | Okunikko, Tochigi | 10/09 /10 | YM | *B. platyphylla* | | Negative | Negative | AB624347 | AB619225 | — | AB619249 |
| Family Rhyparochromidae (outgroup) | | | | | | | | | | | |
| *Togo hemipterus* | Tsukuba, Ibaraki | 17/07 /07 | YK | *Digitaria ciliaris* | Midgut crypts | Negative | Positive | | | | AB619252 |

Abbreviations: COI, mitochondrial *cytochrome oxidase I* gene; *groEL*, bacterial *groEL* gene; *gyrB*, bacterial *gyrB* gene; KK, Katsuyuki Kouno; Negative, PCR-negative amplification; Positive, PCR-positive amplification; 16S rRNA, bacterial 16S rRNA gene; SK, Shuhei Kada; YK, Yoshitomo Kikuchi; YM, Yu Matsuura; TH, Takahiro Hosokawa.

[a]All localities are in Japan.

[b]For example, '11/09/09' means 11 September 2009.

[c]For example, '0% (0/37)' means 0% infection frequency of the symbiont with 0 positive insects of 37 insects examined.

[d]The bacteriocyte-associated endosymbiont of *Nysius* spp.

*P*-values. Pulsed-field gel electrophoresis of the endosymbiont genome was performed with the bacteriome samples dissected from 50 adult insects of *N.* sp. 1 as described (Kikuchi *et al.*, 2009).

*Histological procedures*

Fluorescent *in situ* hybridization was performed with a fluorochrome-labelled oligonucleotide probe Cy3-NpSch1274 (5′-TATACTTTTTGAGGTTCGCTT GCTC-3′) that specifically targets 16S rRNA of the endosymbionts of *Nysius* spp. Adult insects were dissected in phosphate-buffered saline and fixed with Carnoy's solution (ethanol/chloroform/acetic acid = 6:3:1) overnight, and treated with 6% hydrogen peroxide in 80% ethanol for several weeks for quenching autofluorescence of the tissues (Koga *et al.*, 2009). Then, the tissue samples were subjected to *in situ* hybridization as described (Kikuchi *et al.*, 2009). For transmission electron microscopy, adult insects of *N. plebeius* were dissected with fine forceps in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde, and isolated bacteriomes and ovaries were processed into ultrathin sections and observed as described (Fukatsu *et al.*, 2000).

## Results

*General observation of symbiotic organs in* Nysius *spp. and allied lygaeid species*

In both adult females and males of *N. plebeius* (Figure 1a), a pair of large bacteriomes, amorphous in shape and deep red in color, was found in association with their gonads (Figures 1b and c). Although many allied lygaeoid and coreoid species have a characteristic midgut fourth section with a number of crypts for harboring gut symbiotic bacteria (Kikuchi *et al.*, 2011), such a midgut structure was absent in *N. plebeius* (Figures 1b and c). In the ovaries of adult females, a red-pigmented zone, previously designated as 'infection zone' (Schneider, 1940), was observed in each germarium, and the anterior pole of each oocyte was marked by an orange-colored region, wherein a structure so-called 'symbiont ball' was located (Schneider, 1940) (Figure 1d). The bacteriome contained numerous tubular bacterial cells, as did the infection zone of the ovaries (Figures 1e and f). Similar histological and symbiotic configurations were also observed in other *Nysius* species including *N.* sp. 1, *N.* sp. 2 and *N. expressus* (Supplementary Figures S1A–C). These observations agreed with the early report on European *Nysius* species (Schneider, 1940).

In European *Kleidocerys* species, namely *K. resedae* and *K. ericae*, an unpaired, red-colored bacteriome has been identified in association with the midgut (Schneider, 1940; Küchler *et al.*, 2010). We collected samples of *K. resedae* from two distant populations in Japan, Sapporo and Okunikko, and confirmed the same symbiotic architecture: a reddish bacteriome was associated with the midgut,

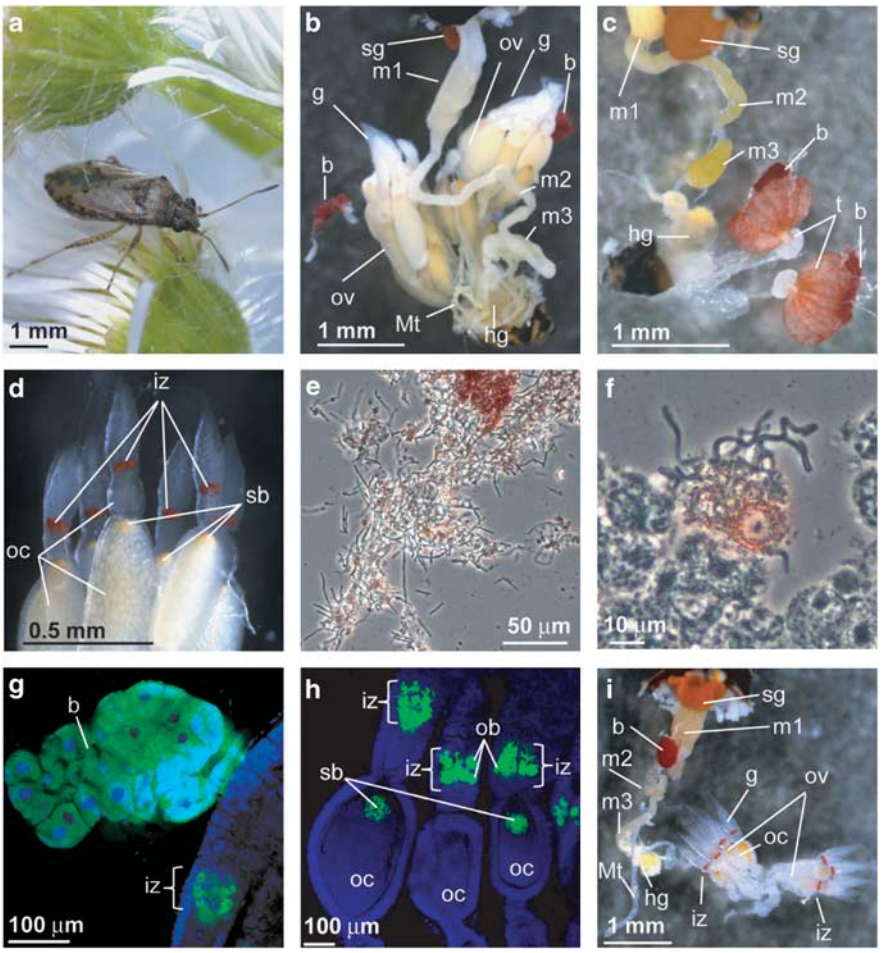


**Figure 1** (**a**) An adult female of *Nysius plebeius*. (**b**) A dorsal view of the dissected abdomen of an adult female of *N. plebeius*. (**c**) A dorsal view of the dissected abdomen of an adult male of *N. plebeius*. Note that large red bacteriomes, the symbiotic organs, are present on both sides of the gonads in both sexes. (**d**) An enlarged image of germaria and developing oocytes in the ovarioles of an adult female of *N. plebeius*. Note a red infection zone in each germarium and an orange region at the anterior pole of each oocyte, the locations of the endosymbiont in the female reproductive system. (**e**) A phase-contrast microscopic image of a crushed bacteriome, in which numerous tubular endosymbiont cells are observed. Note that red materials are seen outside the bacterial cells. (**f**) A similar image of a crushed germarium containing an infection zone. (**g**) Endosymbiont localization in the bacteriome visualized by *in situ* hybridization. (**h**) Endosymbiont localization in the infection zone of the germaria as well as in the symbiont ball at the anterior pole of the oocytes visualized by *in situ* hybridization. In (**g**) and (**h**), green and blue indicate the endosymbionts and the host insect nuclei, respectively. (**i**) A ventral view of the dissected abdomen of an adult female of *Kleidocerys resedae* collected at Sapporo, Japan. Note the presence of a single red-colored bacteriome associated with the midgut, the absence of the paired bacteriome of the *Nysius* type and the presence of the red-colored infection zone in each germarium. b, bacteriome; g, germarium; hg, hind gut; iz, infection zone; m1, midgut first section; m2, midgut second section; m3, midgut third section; Mt, Malpighian tubule; ob, ovarial bacteriocyte; oc, oocyte; ov, ovariole; sb, symbiont ball; sg, stink gland.

whereas no midgut fourth section with crypts was found (Figure 1i).

In the family Lygaeidae, the subfamily Orsillinae, including the genus *Nysius*, the subfamily Ischnorrhynchinae, including the genus *Kleidocerys*, and the subfamily Lygaeinae constitute a well-defined clade (Henry, 1997). However, histological inspection of lygaeine species revealed neither bacteriomes nor midgut fourth section with crypts (Supplementary Figures S1D–F).

### *Electron microscopy of the symbiotic organs of* N. plebeius

Transmission electron microscopy revealed that numerous tubular bacterial cells are harbored in the cytoplasm of bacteriocytes constituting the bacteriome (Figures 2a and b). Figure 2c shows a semi-ultrathin section of the germarium region of an ovariole dissected from an adult female. At the tip of the ovariole, a number of nurse cells were arranged in a corn-like shape, in the center of which a nutritive cord for material transport to oocytes was seen. Following the nurse cell region, an egg chamber region was present, where several incipient oocytes were located centrally, whereas a layer of many follicle cells was located peripherally. In the infection zone region, peculiar round cells, tentatively called ovarial bacteriocytes, were found between the follicle cell layer and the oocytes. Transmission electron microscopy demonstrated

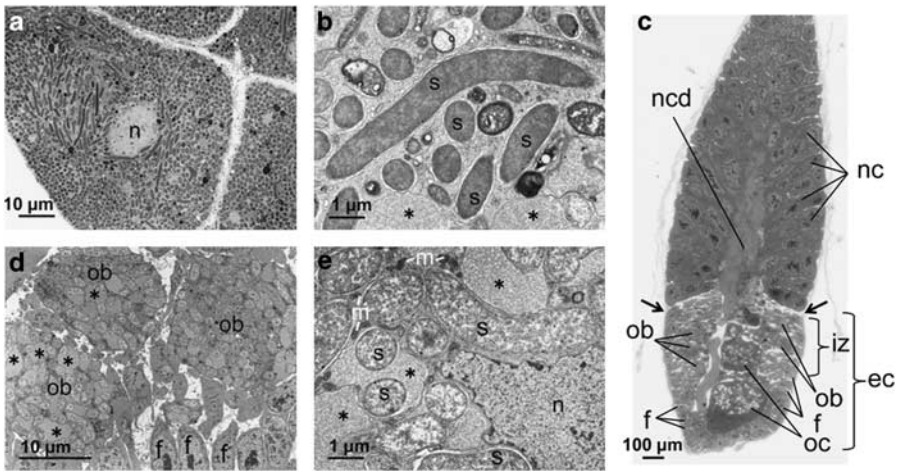


**Figure 2** Transmission electron microscopy of the endosymbiotic system of *Nysius plebeius*. (**a**) Bacteriocytes in a bacteriome whose cytoplasm is filled with tubular endosymbiont cells. (**b**) An enlarged image of the endosymbiont cells in the bacteriome. (**c**) A light microscopic image of semi-ultrathin section of a germarium region of an ovariole, stained with toluidine blue. (**d**) An enlarged image of infection zone in an ovariole, where ovarial bacteriocytes containing many endosymbionts are surrounded by follicular cells. (**e**) An enlarged image of endosymbiont cells in an ovarial bacteriocyte. Asterisks indicate vacuole-like structures that are abundantly found in the ovarial bacteriocytes. b, bacteriocyte; ec, egg chamber; f, follicular cell; iz, infection zone; n, nucleus; nc, nurse cell; ncd, nutritive cord; ob, ovarial bacteriocyte; oc, oocyte; s, endosymbiont cell.

that in the infection zone, the endosymbiont cells were preferentially localized in the ovarial bacteriocytes (Figures 2d and e). In both the bacteriome bacteriocytes and the ovarial bacteriocytes, peculiar vacuoles were frequently associated with the endosymbiont cells (Figures 2b and e, asterisks).

*Bacterial 16S rRNA gene sequences from* Nysius *spp.*
From the bacteriomes of five adult females of *N. plebeius* collected at Tsukuba, a bacterial 16S rRNA gene region was amplified by PCR and cloned, and 7–24 clones for each of the samples were subjected to restriction fragment length polymorphism genotyping. Of the 55 clones examined, 48 clones exhibited the same major pattern, whereas the other 7 clones showed two minor patterns. When three or more clones of the major type were sequenced for each of the samples, all the sequences, 1479 bp in size, were identical to each other, exhibiting the highest BLAST (Basic Local Alignment Search Tool) hit to 16S rRNA gene sequence of secondary endosymbiont of mealybug *Planococcus kraunhiae* (91.4% (1364/1492) sequence identity; accession number AB374417). Meanwhile, the minor clones yielded two α-proteobacterial sequences: one (accession number AB624349) with the highest BLAST hit to 16S rRNA gene sequence of *Wolbachia* endosymbiont of spider mite *Bryobia sarothamni* (99.9% (1426/1427) sequence identity; accession number EU499315), and the other (accession number AB624350) with the highest BLAST hit to 16S rRNA sequence of an uncultured bacterium of the *Rickettsiales* from tick *Ixodes persulcatus* (98.8% (1390/1407) sequence identity; accession number AF493952).

Similarly, bacteriome-derived 16S rRNA gene clones of the major restriction fragment length polymorphism genotype were sequenced for samples of *N. plebeius*, *N.* sp. 1, *N.* sp. 2 and *N. expressus* that represented 8, 5, 1 and 1 populations, respectively. The 1479 bp sequences from *N. plebeius* were 100% identical within the species, and so were the 1421 bp sequences from *N.* sp. 1. Meanwhile, the sequence identities between the bacterial sequences from different *Nysius* species ranged from 98.8% to 99.6%.

Furthermore, bacteriome-derived 16S rRNA gene clones were also sequenced for samples of *K. resedae* collected at Sapporo and Okunikko, Japan. The 1474 bp sequences were almost identical with the exception of a nucleotide site between the populations, exhibiting the highest BLAST hit to 16S rRNA gene sequence of *Kleidoceria schneideri*, the endosymbiont of *K. resedae* sampled in Germany (99.8% (1472/1475) sequence identity; accession number FN555107).

In situ *hybridization of the endosymbiont in* N. plebeius
Dissected tissues of *N. plebeius* were subjected to whole mount *in situ* hybridization with a fluorochrome-labelled probe specifically targeting the endosymbiont 16S rRNA. In adult females, the endosymbiont signals were specifically detected in the bacteriomes, the infection zone of germaria, and the symbiont ball located at the anterior pole of mature oocytes (Figures 1g and h). In adult males, the endosymbiont signals were found in the bacteriomes, but not in the reproductive organs (data not shown).

*Prevalence of the endosymbiont infection in natural populations of* Nysius *spp.*
In total, 151 individuals of *N. plebeius* from 9 populations, 80 individuals of *N.* sp. 1 from 5

populations, 17 individuals of *N.* sp. 2 from 3 populations and 33 individuals of *N. expressus* from 1 population were subjected to diagnostic PCR surveys of their symbiotic bacteria. The bacteriocyte-associated endosymbiont was detected from all the *Nysius* samples representing 4 species, 18 populations and 281 individuals (Table 1). In contrast, *Burkholderia* spp., known as symbiotic associates harbored in the midgut crypts of diverse lygaeoid and coreoid species (Kikuchi *et al.*, 2011), were not detected in any of the *Nysius* samples (Table 1).

*Molecular phylogenetic analysis of the endosymbiont of* Nysius *spp.*

Bacterial 16S rRNA, *groEL* and *gyrB* genes were amplified by PCR, cloned and sequenced from *Nysius* spp. representing 4 species and 16 populations in total (Table 1) and subjected to molecular phylogenetic analyses (Supplementary Figures S2–S4). In all the phylogenies, the endosymbionts of *Nysius* spp. consistently formed a well-defined monophyletic group in the *Gammaproteobacteria*. The phylogenetic relationships reflected the systematics of the host insects: the endosymbionts of *N. plebeius* from different populations formed a clade, and so did the endosymbionts of *N.* sp. 1 from different populations. In the DNA databases, no bacterial sequences were identified as closely related to the endosymbiont clade of *Nysius* spp. In the γ-proteobacterial phylogenies, although statistical supports for the clusters were generally low, the following insect symbionts were placed nearby the endosymbiont clade of *Nysius* spp.: endosymbionts of various insects such as *Blochmannia* of ants, *Wigglesworthia* of tsetse flies, *Buchnera* of aphids and *Baumannia* of sharpshooters; *Kleidoceria* endosymbiont of the birch catkin bug *K. resedae*; and gut symbionts of various pentatomoid stinkbugs such as *Rozenkranzia* of acanthosomatids, *Ishikawaella* of plataspids and *Benitsuchiphilus* of parastrachiids (Supplementary Figures S2–S4).

*Relationship to the endosymbiont of* K. resedae

In the 16S rRNA gene phylogeny, the endosymbiont of *K. resedae* clustered with the endosymbionts of *Nysius* spp., but statistical supports for the grouping were consistently very low (Supplementary Figure S2), suggesting that the apparent clustering is dubious. In the *groEL* and *gyrB* gene phylogenies, the endosymbiont of *K. resedae* did not cluster with the endosymbionts of *Nysius* spp., but was grouped with endosymbionts of other insects such as *Wigglesworthia* of tsetse flies (Supplementary Figures S3 and S4). An approximately unbiased test statistically rejected the hypothesis that the endosymbiont of *K. resedae* constitutes a clade with the endosymbionts of *Nysius* spp. (Table 2).

**Table 2** AU tests for clades to assess the phylogenetic relationships of the endosymbionts of *Nysius* and *Kleidoceria schneideri*

| *Clade* | $\Delta ln\ L$[a] | P-*value* |
|---|---|---|
| (Ksc, Wig) | −12.2 | 0.983 |
| (Ksc, Wig, Blo) | −6.2 | 0.915 |
| (SymNys, Ksc, Wig, Blo) | −5.3 | 0.850 |
| (SymNys, Ksc, Wig) | 6.2 | 0.282 |
| (SymNys, Bau) | 6.7 | 0.094 |
| (SymNys, Blo) | 6.9 | 0.224 |
| (SymNys, Ben, Buc) | 8.1 | 0.789 |
| (SymNys, Ksc) | 20.2 | 0.024[b] |
| (SymNys, Wig) | 21.1 | 0.017[b] |
| (SymNys, Buc) | 26.3 | 0.051 |
| (SymNys, Ben) | 27.7 | 0.021[b] |

Abbreviations: AU test, approximately unbiased test; Bau, *Baumannia cicadellinicola*; Ben, *Benitsuchiphilus tojoi*; Blo, *Blochmannia floridanus* and *B. pennsylvanicus*; Buc, *Buchnera aphidicola* (aphid *Acyrthosiphon pisum*) *and B. aphidicola* (aphid *Schizaphis graminum*); Ksc, *Kleidoceria schneideri*; SymNys, endosymbionts of *Nysius plebeius* (Tsukuba) and *N. expressus*; Wig, *Wigglesworthia glossinidia*.
[a]Difference of log-likelihood between the best trees among those that reject and support the monophyly of the clade.
[b]The clade is significantly rejected ($P<0.05$).

*Molecular phylogenetic analysis of* Nysius *spp. and allied lygaeid stinkbugs*

Mitochondrial *COI* gene was amplified by PCR and sequenced from *Nysius* spp. and allied stinkbug species of the family Lygaeidae, and subjected to molecular phylogenetic analyses. The phylogenetic relationship agreed with the systematics of the lygaeid stinkbugs: the three subfamilies respectively formed well-defined monophyletic groups in the Lygaeidae, and congenic species also respectively constituted monophyletic groups in the phylogeny. *Nysius* spp. and *K. resedae* did not form a clade (Supplementary Figure S5).

*Co-speciating pattern between* Nysius *spp. and their endosymbionts*

Figure 3 contrasts the phylogeny of *Nysius* spp. inferred from mitochondrial *COI* gene sequences with the phylogeny of their endosymbionts inferred from concatenated sequences of bacterial 16S rRNA, *groEL* and *gyrB* genes. The topology of the host insect phylogeny was congruent with the topology of the endosymbiont phylogeny.

*Molecular evolutionary and genomic aspects of the endosymbionts of* Nysius *spp.*

Nucleotide compositions of 16S rRNA, *groEL* and *gyrB* genes of the *Nysius* endosymbionts were AT-rich, 51.1–51.4%, 63.3–64.5% and 70.4–71.8%, in comparison with those of free-living γ-proteobacteria at the values around 44–46%, 42–50% and 40–52%, respectively (Supplementary Figures S2–S4). Evolutionary rates of 16S rRNA, *groEL* and *gyrB* genes of the *Nysius* endosymbionts were significantly higher than those of free-living γ-proteobacteria such as *Escherichia coli* and *Pantoea* spp.,

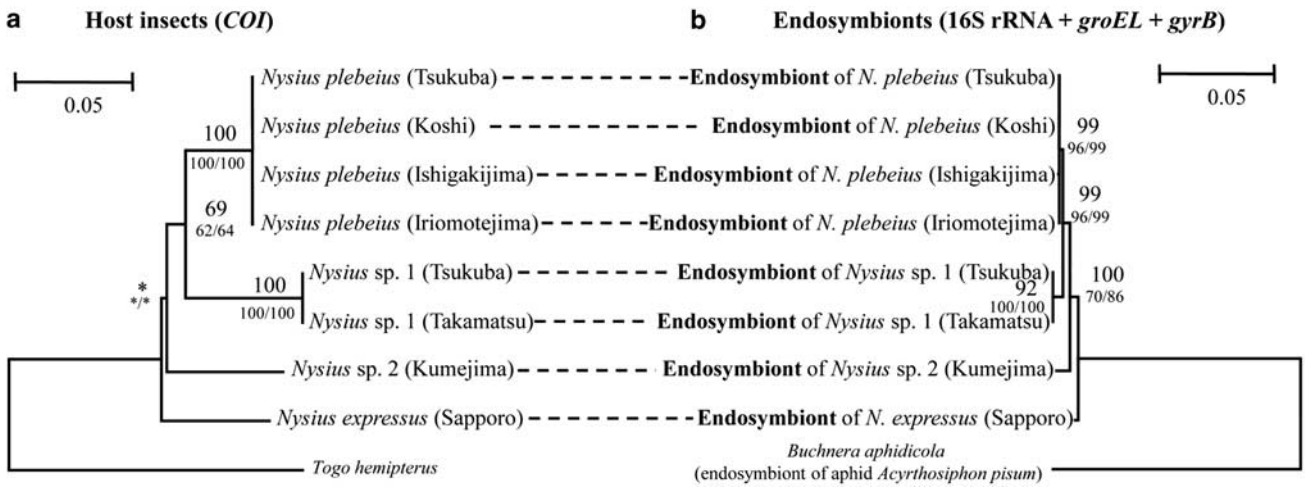


**Figure 3** Phylogenetic concordance between the *Nysius* species and their endosymbionts. (**a**) A maximum likelihood phylogeny of the host insects inferred from mitochondrial *COI* gene sequences (558 aligned nucleotide sites). (**b**) A maximum likelihood phylogeny of the endosymbionts inferred from concatenated sequences of 16S rRNA, *groEL* and *gyrB* genes (2876 aligned nucleotide sites).

**Table 3** Relative rate tests for comparing the molecular evolutionary rates of 16S rRNA, *groEL* and *gyrB* gene sequences between the lineages of the endosymbiont of *Nysius plebeius*, its free-living relatives and *Buchnera aphidicola,* the obligate endosymbionts of aphids

| *Gene* | *Lineage 1* | *Lineage 2* | *Outgroup* | *K1*[a] | *K2*[b] | *K1–K2* | *Rate ratio*[c] | P-*value*[d] |
|---|---|---|---|---|---|---|---|---|
| 16S rRNA | Endosymbiont of *N. plebeius* (AB576918) | *Escherichia coli* (U00096) and *Pantoea agglomerans* (AY691545) | *Vibrio cholerae* (CP001485) | 0.068 | 0.028 | 0.040 | 2.4 | $3.6 \times 10^{-5}$ |
| *groEL* | Endosymbiont of *N. plebeius* (AB583951) | *E. coli* (U00096) and *P. agglomerans* (AB008142) | *V. cholerae* (CP001485) | 0.094 | 0.030 | 0.064 | 3.1 | $1.9 \times 10^{-7}$ |
| *gyrB* | Endosymbiont of *N. plebeius* (AB583935) | *E. coli* (U00096) and *P. ananatis* (NC_013956) | *V. cholerae* (CP001485) | 0.212 | 0.060 | 0.152 | 3.5 | $1.3 \times 10^{-7}$ |
| 16S rRNA | Endosymbiont of *N. plebeius* (AB576918) | *Buchnera aphidicola* str. APS (NC_002528) and *B. aphidicola* str. Sg (NC_004061) | *V. cholerae* (CP001485) | 0.053 | 0.066 | −0.013 | 0.80 | 0.28 |
| *groEL* | Endosymbiont of *N. plebeius* (AB583951) | *B. aphidicola* str. APS (NC_002528) and *B. aphidicola* str. Sg (NC_004061) | *V. cholerae* (CP001485) | 0.073 | 0.069 | 0.004 | 1.1 | 0.79 |
| *gyrB* | Endosymbiont of *N. plebeius* (AB583935) | *B. aphidicola* str. APS (NC_002528) and *B. aphidicola* str. Sg (NC_004061) | *V. cholerae* (CP001485) | 0.133 | 0.167 | −0.034 | 0.80 | 0.21 |

[a]Estimated mean distance between lineage 1 and the last common ancestor of lineages 1 and 2.
[b]Estimated mean distance between lineage 2 and the last common ancestor of lineages 1 and 2.
[c]K1/K2.
[d]*P*-values are estimated by the program RRTree (Robinson-Rechavi and Huchon, 2000). The numbers of nucleotide sites used are 1224, 1010 and 640 for 16S rRNA, *groEL* and *gyrB* genes, respectively.

whereas the evolutionary rates were almost equivalent to those of aphid endosymbiont *Buchnera* (Table 3). Pulsed-field gel electrophoresis of the bacteriomes of *N.* sp. 1 estimated the genome size of the endosymbiont as 0.6 Mb (Supplementary Figure S6).

## Discussion

From these results, we conclude that: (1) seed bugs of the genus *Nysius* are associated with a specific clade of γ-proteobacterial endosymbionts (Supplementary Figures S2–S4); (2) the endosymbiont is endocellularly localized in specific organs, bacteriomes, associated with host gonads of both sexes (Figures 1b, c and g); (3) in female ovaries, the endosymbiont is also localized in 'infection zone' in the middle of germaria as well as in 'symbiont ball' at the anterior pole of oocytes (Figures 1d and h), indicating vertical transmission of the endosymbiont via the ovarial passage; (4) the endosymbiont does not form a clade with *K. schneideri*, the endosymbiont of birch catkin bug *K. resedae* (Supplementary Figures S2–S4 and Table 2), suggesting multiple evolutionary origins of the bacteriome-associated endosymbionts in the family Lygaeidae; (5) the endosymbiont genes exhibit

AT-biased nucleotide compositions and accelerated rates of molecular evolution (Supplementary Figures S2–S4 and Table 3), and the endosymbiont genome is as small as 0.6 Mb in size (Supplementary Figure S6), indicating a remarkable reductive genome evolution in the endosymbiont lineage; and (6) the phylogenetic relationship of the endosymbionts agrees with the phylogenetic relationship of the host insects (Figure 3), suggesting an intimate host–symbiont association over evolutionary time.

*Host–symbiont co-evolution in the genus* Nysius

Thus far, in diverse insect endosymbioses of obligate nature, it has been shown that the phylogenetic relationship of the endosymbionts generally agrees with the phylogenetic relationship of their hosts (Moran *et al.*, 1993, 2005; Chen *et al.*, 1999; Sauer *et al.*, 2000; Thao *et al.*, 2000; Thao and Baumann, 2004; Takiya *et al.*, 2006; Conord *et al.*, 2008). In some stinkbug lineages, such co-cladogenetic patterns with their obligate gut symbionts have been observed (Hosokawa *et al.*, 2006; Kikuchi *et al.*, 2009). Our results suggest that in the seed bug genus *Nysius*, the endosymbionts and their hosts have experienced such a co-evolutionary history: the endosymbiont was already present in the common ancestor of the *Nysius* species and has been subjected to strict vertical transmission and co-speciation over evolutionary time (Figure 3). However, it should be noted that we cannot rule out the possibility that the apparent phylogenetic congruence could have occurred by chance. Considering only 15 possible rooted tree topologies for four species, the chance that the symbiont tree will exactly match the host tree is expected to be 6.7%, which is not a negligible value. To confirm the validity of the host–symbiont co-speciation, therefore, more *Nysius* species should be added to the analysis.

*Reductive genome evolution of the endosymbiont of* Nysius *spp.*

The AT-biased nucleotide compositions and the accelerated evolutionary rates of the endosymbiont genes (Supplementary Figures S2–S4 and Table 3), and reduced genome size of the endosymbiont (Supplementary Figure S6), are also supportive of the long-lasting host–symbiont co-evolution. The estimated genome size, 0.6 Mb, is strikingly smaller than those of free-living γ-proteobacteria like *E. coli* (4.6 Mb) and *Vibrio cholerae* (4.0 Mb) (Blattner *et al.*, 1997; Heidelberg *et al.*, 2000), relatively smaller than those of obligate gut symbionts of other stinkbugs like *Ishikawaella* of plataspids (0.82–0.83 Mb) and *Rosenkranzia* of acanthosomatids (0.90–0.96 Mb) (Hosokawa *et al.*, 2006; Kikuchi *et al.*, 2009), and as small as those of obligate endosymbionts of other insects like *Buchnera* of aphids (0.42–0.65 Mb) and *Wigglesworthia* of tsetse flies (0.70 Mb) (Shigenobu *et al.*, 2000; Akman *et al.*, 2002). It has been argued that such evolutionary patterns may be attributed to stable and nutrition-rich endocellular habitat for the endosymbiont and also attenuated purifying selection because of the small population size and strong bottleneck associated with the lifestyle of the vertically transmitted endosymbiont (Wernegreen 2002; Moran *et al.* 2008).

*Biological function of the endosymbiont*

The biological function of the endosymbiont for the *Nysius* seed bugs is currently unknown. However, considering the highly developed endosymbiotic system (Figure 1), the 100% infection frequencies in natural host populations (Table 1), the presumably long-lasting host–symbiont association (Figure 3) and the essential roles of the gut symbionts in other stinkbug groups (Abe *et al.*, 1995; Fukatsu and Hosokawa, 2002; Hosokawa *et al.*, 2006; Kikuchi *et al.*, 2007, 2009; Prado and Almeida, 2009a, b), it appears likely that the endosymbiont also plays some important biological roles in *Nysius* spp. Plausibly, the endosymbiont may supply nutritional supplements for the hosts, such as essential amino acids and vitamins, as has been demonstrated for other plant-sucking insects (Douglas, 1998; Baumann, 2005).

*Transmission route of the endosymbiont*

The localization of the endosymbiont at the anterior pole of oocytes, which comprises the structure called 'symbiont ball' (Schneider, 1940) (Figures 1d and h), indicates vertical transmission of the endosymbiont via the ovarial passage through host generations. At the tip of each ovariole, the endosymbiont signals were detected at a distinct, zone-like and red-colored region in the middle of germarium (Figures 1d and h), the structure referred to as 'infection zone' (Schneider, 1940). In the fruitfly *Drosophila melanogaster*, a zonal region in the middle of each germarium, which represents the somatic stem cell niche, was shown to be the entry point of a *Wolbachia* endosymbiont into the germline (Frydman *et al.*, 2006). Whether the infection zone observed in the ovary of *Nysius* species corresponds to the somatic stem cell niche in the ovary of *Drosophila* is of interest and deserves further studies. At present, our electron microscopic observations suggest that the endosymbiont cells are not accumulated in the presumable somatic stem cell niche (Figure 2c, arrows) but localized in the ovarial bacteriocytes (Figures 2d and e). Although vertically transmitted endosymbionts of diverse insects tend to localize at the posterior pole of host eggs where the germ plasm destined to form germline cells is located (Buchner, 1965; Miura *et al.*, 2003; Veneti *et al.*, 2004), some insects, including coccids, lygaeid bugs and others, exceptionally exhibit endosymbiont localization at the

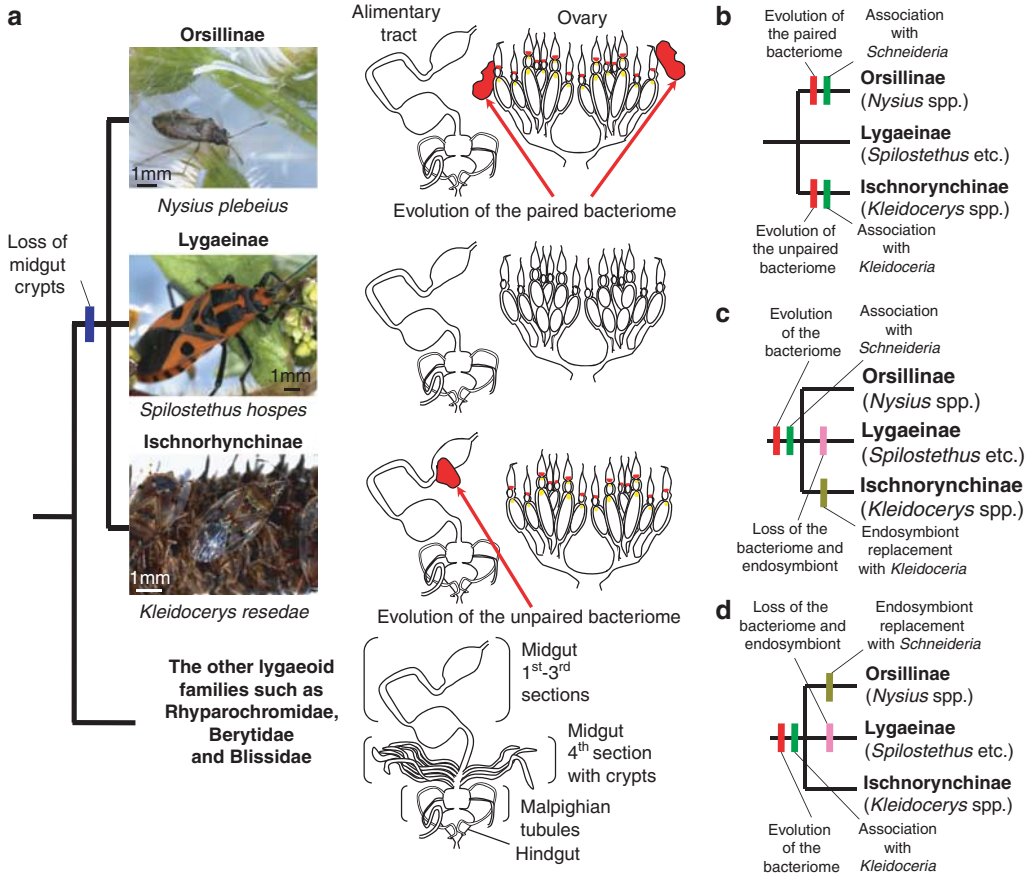


**Figure 4** Evolution of symbiotic organs and endosymbionts in the Lygaeidae. (**a**) Symbiotic organs in the lygaeid subfamilies Orsillinae, Lygaeinae and Ischnorhynchinae. (**b–d**) Alternative hypotheses that account for the evolution of the bacteriomes and the endosymbionts in the Lygaeidae.

anterior pole of their eggs (Schneider, 1940; Buchner, 1965; Fukatsu and Nikoh, 2000; Küchler *et al.*, 2010).

*Pigmentation of the symbiotic organs*

The symbiotic organs of *Nysius* spp., namely the bacteriome as well as the infection zone in the ovary, are vividly colored in red (Figures 1b–d). Smeared specimens of the bacteriome and the infection zone revealed that the endosymbiont cells are not colored but some materials derived from the host cytoplasm are red in color (Figures 1e and f). Electron microscopic images of the infection zone showed that amorphous vesicles are frequently associated with the endosymbiont cells (asterisks in Figures 2b and e), which might be related to the accumulation of the red pigment in the symbiotic organs. It is currently unknown whether the endosymbiont or the host produces the red pigment and what is the biological relevance of the red pigment. Interestingly, the bacteriome and the infection zone of a distinct lygaeid species *K. resedae* are also colored in red (Figure 1i) (Schneider, 1940; Küchler *et al.*, 2010). The stink gland and the testis of these insects were also reddish in color (Figures 1b, c and i and Supplementary Figure S1), but diverse stinkbug groups generally exhibit these morphological traits. The colocalization of the red pigment with the endosymbiont will be useful in tracing the developmental dynamics of the bacteriome-associated endosymbiosis in these lygaeid stinkbugs.

*Evolution of the bacteriomes and the endosymbionts in the Lygaeidae*

In the family Lygaeidae, the subfamilies Orsillinae (*Nysius* spp. and others), Lygaeinae (*Spilostethus* spp., *Oncopeltus* spp. and others) and Ischnorhynchiinae (*Kleidocerys* spp. and others) form a monophyletic group (Supplementary Figure S5 and Table 1) (Henry, 1997). All members of the subfamilies examined thus far lack the midgut fourth section with crypts (Figures 1b, c and i and Supplementary Figure S1) (Kikuchi *et al.*, 2011), suggesting that their common ancestor had already lost the midgut crypts (Figure 4a). On the grounds that the endosymbiont clade of *Nysius* species is phylogenetically distinct from the endosymbiont of *Kleidocerys* species (Supplementary Figures S2–S4 and Table 2), and that the paired bacteriome of *Nysius* species anatomically look quite different

from the unpaired bacteriome of *Kleidocerys* species (Figure 4a), it seems plausible that the bacteriomes have independently evolved in the *Nysius* lineage and the *Kleidocerys* lineage, respectively (Figure 4b). On the other hand, the red color of the bacteriomes, the red-colored infection zone in the germaria and the symbiont ball at the anterior pole of oocytes are commonly observed in the *Nysius* species and the *Kleidocerys* species (Figure 4a), which favors an alternative hypothesis that the bacteriome and the endosymbiont evolved in the common ancestor and subsequently lost in the lineage of Lygaeinae, while a bacteriome remodeling and an endosymbiont replacement have occurred either in the *Nysius* lineage or in the *Kleidocerys* lineage (Figures 4b and c). At present, it is elusive which of these or other evolutionary hypotheses are to be substantiated. In the family Blissidae that is allied to the Lygaeidae, *I. sabuleti* was reported to possess a pair of bacteriomes in the fat body (Schneider, 1940). Future studies on the *Ischnodemus* system would shed light on the endosymbiotic evolution in the Lygaeidae. Recently, from the bulrush bug *Chilacis typhae* (Lygaeoidea: Artheneidae), a γ-proteobacterial endosymbiont was characterized in strongly enlarged epithelial cells at an anterior region of the midgut (Küchler *et al.*, 2011). On the grounds that (1) the endosymbiont is phylogenetically not close to the endosymbionts of *Nysius* spp. and *Kleidocerys* spp., (2) the histological configuration of the symbiotic organ is totally different from the symbiotic organs of *Nysius* spp. and *Kleidocerys* spp. and (3) the host insect is also phylogenetically not close to *Nysius* spp. and *Kleidocerys* spp. (Henry, 1997), it seems likely that the endosymbiotic system in *C. typhae* has an evolutionary origin distinct from the endosymbiotic systems in *Nysius* spp. and *Kleidocerys* spp.

*Proposal of candidate name*

On account of these distinct and coherent microbiological, phylogenetic and evolutionary traits described in this study, we propose the designation '*Candidatus* Schneideria nysicola' for the endosymbiotic bacterial clade associated with seed bugs of the genus *Nysius*. The generic name honors Gerhard Schneider, who first described the bacteriome and the endosymbiont of *Nysius* spp. (Schneider, 1940). The specific name indicates the association with *Nysius* seed bugs.

*Perspectives*

The bacteriome, a novel organ specialized for harboring endosymbiotic microorganisms, is of evolutionary and developmental interest. To gain insights into the enigma, the *Nysius* seed bugs and their relatives will offer ideal study systems for the following reasons: (1) in the family Lygaeidae, the bacteriome may have evolved twice in the lineages of *Nysius* spp. and *Kleidocerys* spp.; (2) the Lygaeidae also embraces stinkbugs without the bacteriome such as *Spilostethus, Graptostethus* and *Tropidothorax* species; (3) therefore, morphological, developmental and molecular biological comparisons between allied species with and without the symbiotic organ are feasible; (4) in *Nysius plebeius* and *Oncopeltus fasciatus*, it has been shown that RNA interference works efficiently (Hughes and Kaufman, 2000; Futahashi *et al.*, 2011), which enables molecular genetic approaches to the mechanisms as to what host genes are involved in the endosymbiosis and how the symbiotic organ is differentiated and formed; and (5) we have established rearing techniques for the *Nysius, Kleidocerys, Spilostethus* and *Graptostethus* species in Petri dishes on plant seeds, which offer tractable model systems for experimental and functional studies. Thus far, the aphid–*Buchnera* association has been the best-studied model endosymbiotic system with the bacteriome, wherein both the host genome and the endosymbiont genome have been sequenced (Shigenobu *et al.*, 2000; International Aphid Genomics Consortium, 2010a), developmental processes of the bacteriome formation have been histologically documented (Braendle *et al.*, 2003; Miura *et al.*, 2003), the transcriptomics of the symbiotic organ have been conducted (Nakabachi *et al.*, 2005; Hansen and Moran, 2011) and a number of physiological, ecological and molecular works on the endosymbiosis have been accumulated (reviewed in Douglas, 1998; Baumann, 2005). On the other hand, as almost all species of the family Aphididae possess the bacteriome harboring *Buchnera* endosymbiont and a few exceptional species without the bacteriome represent secondary losses of the symbiotic system (Fukatsu *et al.*, 1994; Braendle *et al.*, 2003), evolutionarily meaningful comparisons between allied aphid species with and without the symbiotic organ are difficult. In aphids, it has been reported that RNA interference generally does not work efficiently (Jaubert-Possamai *et al.*, 2007; International Aphid Genomics Consortium, 2010b), which has hindered approaches to the molecular mechanisms underlying the endosymbiotic association. Genome sequencing of the *Schneideria* endosymbiont and transcriptomics of the bacteriome of the *Nysius* host will, in combination with the laboratory rearing protocols and the RNA interference techniques, provide a complementary and promising model system for insect symbiosis studies.

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